Examining the Effects of Ingested *Deepwater Horizon* Oil on Juvenile Red-Eared Sliders (*Trachemys scripta elegans*) and Common Snapping Turtles (*Chelydra serpentina*) as Surrogate Species for Sea Turtles

Technical Report
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Executive Summary

Hundreds of externally and internally oiled juvenile oceanic sea turtles were documented and rescued during the *Deepwater Horizon* (DWH) oil spill. Polycyclic aromatic hydrocarbon (PAH) metabolites in the bile and tissues of some individuals confirmed internal exposure and metabolism of oil. Although the adverse effects associated with becoming physically mired in oil were readily apparent in sea turtles rescued during the DWH spill, there are limited data on potential effects of oil ingestion in sea turtles. Therefore, a 14-day, controlled-dose ingestion laboratory study was undertaken using DWH oil (MC 252) to examine potential effects of internal oil exposure on common species of freshwater turtles, red-eared sliders, and the common snapping turtle as surrogates for endangered sea turtles. Estimated amounts of oil in the oral cavities of turtles rescued from the field during the DWH spill were used to calculate environmentally relevant daily doses for this study. Numerous chemical, biological, and physiological endpoints were assessed, including those important for general clinical assessment, as well as additional endpoints known to respond to acute exposure to PAHs in other taxa (e.g., oxidative stress, DNA damage). Specifically, effects on the hypothalamic-pituitary-adrenal (HPA) axis, which regulates stress response and other vital functions, were investigated due to observations of potential HPA disruption in other vertebrate species exposed to DWH and other crude oil.

Overall, red-eared sliders and snapping turtles orally exposed to MC 252 oil for 14 days during the surrogate study did not show mortality or severe life-threatening physiological derangements. Dose-dependent increases in bile metabolites demonstrated that turtles had metabolized oil constituents. Oil-related alterations in food assimilation, malabsorption, and dehydration were observed. Non-statistically significant alterations of HPA axis function were also observed. Some antioxidant levels and measures of oxidative stress and DNA damage showed changes that were consistent with PAH exposure. However, there was an inability to dose surrogate turtles for time periods comparable to other studies where such effects were shown in orally dosed vertebrates. Delayed and longer-term effects of these low-dose oil exposures, as well as exposure via multiple routes as observed on sea turtles rescued from oiled surface habitats during the DWH oil spill, also were not part of the study design due to logistical constraints.

1. Introduction

Hundreds of externally oiled juvenile oceanic sea turtles were documented and rescued during the *Deepwater Horizon* (DWH) oil spill (Stacy, 2012). Various degrees of external oiling (dermal exposure) were observed, many of these turtles had ingested oil, and exposure likely occurred via inhalation as well (DWH Trustees, 2015, Section 4.8, Sea Turtle Injury). Five categories of external oiling were assigned to these turtles with category 0 being no visible oil, category 1 minimally oiled, category 2 lightly oiled, category 3 moderately oiled, and category 4 heavily oiled (Stacy, 2012). In addition to external oiling, coating oil was also observed in turtle oral cavities, with the frequency of detection and quantity estimated correlated positively with the degree of external oiling observed (Mitchelmore et al., 2015). Internal exposure was confirmed by the detection of polycyclic aromatic hydrocarbon (PAH) metabolites in bile, and PAHs in tissues from necropsied animals far exceeded those measured in non-oiled sea turtles found within the spill area (Ylitalo et al., 2014). Although the adverse effects associated with becoming physically mired in oil were readily apparent in sea turtles recovered during the DWH spill (Stacy, 2012), there are limited data on potential effects of petroleum ingestion in sea turtles (for a review, see Shigenaka, 2003). Given the large number of sea turtles observed to have consumed various amounts of oil during the DWH spill, an understanding of the resulting health effects was necessary to more fully ascertain the negative consequences of exposures that occurred during the spill.

Therefore, to determine sub-lethal effects of oil ingestion, a 14-day, controlled-dose ingestion laboratory study was undertaken using DWH oil (MC 252) using an array of chemical, biochemical, cellular, and physiological endpoints. The estimated amounts of oil in the oral cavities of turtles from the field were used to calculate environmentally relevant daily doses (Mitchelmore, 2012a, 2012b). However, even though the calculated daily dose was based on field observations, there are still a number of uncertain variables associated with the nature (i.e., multiple routes of exposure), duration, extent, and number of exposures that sea turtles experienced during and after the DWH oil spill.

Because the endangered status of sea turtles precluded their use as subjects, two surrogate freshwater species – red-eared sliders (*Trachemys scripta elegans*) and common snapping turtles (*Chelydra serpentina*) – were selected to encompass disparate phylogenetic, ecological, physiological, and behavioral traits. The red-eared slider (family Emydidae) are a common species of freshwater turtle that are routinely used in laboratory studies as they are easily accessible and easy to maintain in captivity. A second surrogate species, the common snapping turtle (family Chelydridae), were also used. These species exhibit differences in metabolism and susceptibility to some organic contaminants (Eisenreich, 2011; Eisenreich et al., 2012). The use of two surrogate species allowed for comparison of species-specific sensitivity and response to oil, and potentially could provide a more detailed and mechanistic analysis of oil toxicity to

apply to possible effects on sea turtles, assuming that basic biochemical and metabolic processes are conserved.

Numerous chemical, biological, and physiological endpoints were selected for this study, including those important for general clinical assessment, as well as some endpoints known to respond to acute exposure to PAH in other taxa. Examples from this latter category included endpoints of oxidative stress (e.g., alterations in antioxidant level and oxidative damage to lipids) (Mitchelmore et al., 2006), DNA damage (Mitchelmore and Chipman, 1998; Mitchelmore et al., 1998), and assessment of hypothalamic-pituitary-adrenal (HPA) axis function (Mohr et al., 2010). Specifically, effects on the HPA axis were investigated because potential HPA disruptions related to oil exposure have been documented in several vertebrate taxa [e.g., bottlenose dolphins (*Tursiops truncatus*) in Barataria Bay following the DWH spill (Schwacke et al., 2014), sparrows dosed with DWH oil (Lattin et al., 2014), and mink exposed to oil (Mohr et al., 2010)]. Few studies have investigated responses to stress induction, either physical (i.e., handling stress) or chemical via injection of adrenocorticotrophic hormone (ACTH), in turtles (e.g., Cash et al., 1997).

The overall objective of this study was to determine toxicological effects on turtles following a 14-day oral exposure to DWH oil at comparable doses to those that sea turtles were estimated to have been exposed to during the DWH oil spill (Mitchelmore et al., 2015). Although the focus of this study was constrained to acute effects, translation of some of cellular and sub-cellular irregularities after oil exposure may have long-term or even delayed consequences on the overall growth, fitness, and survival of individuals; however, chronic effects were beyond the scope of this study. An additional objective was to investigate and identify biological endpoints that may be suitable for injury and recovery assessments and in future oil spill events.

2. Experimental Design and Methods

Detailed descriptions of the methods can be found in the project work plan (see Appendix A) and are briefly summarized in this report.

2.1 Turtle Husbandry and Care

One-month-old hatchling snapping turtles [~ 1" straight carapace length (SCL)] and red-eared sliders (4" SCL) were purchased from a commercial farm and acclimatized for several months prior to the study in a climate-controlled facility with exposure to ultraviolet (UV) light and fed a complete pelleted ration (TeraFauna ReptoMin®) and meal worms. During the dosing phase, turtles were individually housed in 10-gallon glass tanks in one of two temperature-controlled water baths (18' by 6' fiberglass shallow tanks), in which water temperatures were maintained

between 77°F and 79°F (see Appendix A for further details). UVA/UVB strip lights (on a 10:14 hour light:dark regime) and basking heat ceramic bulbs were placed above the water baths to maintain appropriate light cycle quality and air temperatures. Turtles were offered live mealworms daily during the dosing studies. All turtles were visually examined, food intake and defecation were monitored, and tanks were cleaned daily. Institutional Animal Care and Use Committee (IACUC) approval (IACUC # F-CBL-12-06) was obtained for animal collection and housing at the Chesapeake Biological Laboratory.

2.2 Oil Dosages, Administration, and Monitoring

Red-eared sliders and snapping turtles were tested in separate experiments; red-eared sliders in April 2014 and snapping turtles in May 2014. Surgically inserted esophageal feeding tubes (e-tubes) placed directly into the stomach were used for dosing individuals to provide an accurate dose and reduce the stress and injury caused by daily oral intubation (gavage feeding). The tubes were placed under general anesthesia (Propofol) and turtles were allowed to recover for at least 48 hours prior to dosing. Lymph contamination was a persistent problem during blood collection from peripheral vessels, and likely would have affected the comparability of corticosterone concentration and other blood parameters; therefore, a 1-mm port was drilled in the ventral plastron in the red-eared sliders while they were under anesthesia to allow for blood collection by cardiocentesis. The port was covered by a sterile, waterproof bandage until use. Cardiocentesis was also used for blood collection in the snapping turtles but a port was not required to be drilled as direct access could be achieved through the more flexible plastron.

Each turtle had a health monitoring data sheet assigned to it to keep track of daily health (i.e., feeding, behavior, other visual observations including signs of stress), as well as an experimental monitoring data sheet to record experimental conditions such as daily feeding (i.e., amount, type, and time), daily cleaning, and temperature and light cycles. The turtles remained in their individual tanks for the duration of the experiment, unless e-tubes were removed, which warranted early necropsy of the individual.

Turtles (n = 16–18 per dose group) were exposed daily to a control, low, or high sublethal oil dose (i.e., at 0, 100, 1,000 mg kg^{-1} body weight concentrations, respectively) for a 14-day period. The oil doses represented low to medium estimates of exposure based upon estimated volumes of oil contained in the oral cavity of sea turtles captured during the DWH spill (Mitchelmore et al., 2015). Every day, at the beginning of the test, and throughout the 14-day exposure period, all turtles were briefly removed from their 10-gallon tanks (for approximately 2 min), their body wet weight (WW) and SCL measurements taken, and each turtle was dosed with the appropriate volume of oil based on the turtle's treatment group and weight. To dose turtles, Slick A oil was mixed with a food slurry of powdered Emeraid (Omnivore feed) drawn into a syringe and injected into the e-tube. A bolus of 0.9% sterile saline flushed the tube and ensured that the dose had reached the stomach (see Appendix A for specific details). At all times, weights and lengths

of the turtles, and weights of the oil, feed, and saline doses were recorded on daily dosing data sheets. Turtles were inspected daily for signs of feeding, regurgitation, feces production, and any other signs of stress or discomfort, especially issues related to the e-tube surgical site.

2.3 ACTH Challenge

Following the 14-day dosing regimen, a subset of 10 animals from each treatment group was randomly selected for ACTH stimulation. Those turtles not given ACTH were used as within-treatment controls for analysis of this endpoint to examine whether ACTH affected other endpoints. The dosage and timing of post-stimulation sample collection were chosen based on the results of a pilot study using 32 red-eared sliders and 24 snapping turtles. This pilot study established that an injection of 100 IU (International Units) ACTH/kg body weight for one hour was optimal for a stress response in both surrogate turtle species (Dr. Carys Mitchelmore, University of Maryland Chesapeake Biological Laboratory, personal communication, February 2014).

In the definitive study, all turtles were dosed at approximately the same time of the day to help in the control of any ACTH time-of-day complications. Before dosing, a pre-ACTH blood sample was collected via cardiac puncture using a heparinized needle and syringe to obtain baseline corticosterone levels and for analysis of chemical and biological endpoints (see Appendix A for specific methods). A post-stimulation blood sample was collected 60 min after intracoelomic injection of 50 IU/kg ACTH (porcine pituitary; Sigma Chemical Company, St. Louis, MO) diluted in 0.9% saline.

2.4 Euthanasia, Necropsy, and Blood/Tissue Collection and Processing

Turtles were euthanized by rapid decapitation and pithing of the brain according to the methods outlined in Appendix A. A complete necropsy was performed on all animals and tissues collected and processed as described in Appendix A. The order in which samples were collected was selected to minimize cross-contamination and postmortem interval, which can affect more sensitive assays. Bile (if present) was collected for chemical analysis of PAH metabolites immediately after removal of the plastron. After aspiration of the gall bladder, the whole liver was removed, weighed, and immediately sub-sampled from the right (non-gall bladder containing) lobe for DNA damage assessment by the COMET assay. A Hepatic Somatic Index (HSI) was calculated as liver weight divided by body weight. Other liver samples were placed into cryovials, flash frozen in liquid nitrogen (or dry ice), and stored at -80°C for oxidative stress endpoints [i.e., glutathione (GSH) and lipid peroxidation]. The gastrointestinal (GI) tract was removed intact and segmentally incised at regular intervals (at least five times) to allow

examination of the contents and mucosa, and to facilitate fixation. The adrenal glands were measured (maximum length and width) using calipers. Sections of all other organs and tissues were preserved in 10% neutral phosphate-buffered formalin for histopathology.

Pre-ACTH blood draws were used for all blood chemistry and hematology assessments. If collected, post-ACTH blood was collected and processed for corticosterone analyses only. A portion of whole blood was processed to prepare plasma, which was flash-frozen in liquid nitrogen and stored at -80°C. Plasma preparations were divided into at least three aliquots to send for different analyses [i.e., corticosterone analyses (Cornell University), plasma proteins and blood chemistry panels (University of Miami, FL), and oxidative stress endpoints (University of Maryland Center for Environmental Science)]. A portion of whole heparinized blood was used to determine packed cell volume (PCV; see Appendix A) and to prepare blood smears (see Appendix A) that were assessed for white blood cell (WBC) estimates, and WBC and red blood cell (RBC) morphologic assessments. Additional volumes of whole heparinized blood were also sent to the University of Miami (FL) for PCV assessments and hemoglobin quantification.

2.5 Veterinary Assessment Method and Approaches

2.5.1 Hematology

Complete blood counts (CBCs) were performed by hemacytometer using Natt & Herrick's stain (VetLab Supply, Miami, FL) for total WBC and RBC counts. PCV was determined by centrifugation of a partially filled capillary tube for 8 min in an Autocrit Ultra 3 centrifuge (Becton Dickinson, Franklin Lakes, NJ) and plasma color was reported (e.g., normal, lipemia, hemolysis). Peripheral blood smears were stained with Diff Quik (VWR, Radnor, PA) and examined under oil immersion (1,000x) for WBC differential and detailed morphological review of erythrocytes, leukocytes, and thrombocytes. In addition, one new methylene blue (NMB; Sigma-Aldrich®, St. Louis, MO) stained smear from each blood sample was prepared after incubating whole blood with the stain for 30 min. The NMB-stained slide was evaluated for erythrocyte morphology.

Hemoglobin was determined using a method adapted for avian samples on the Hemavet analyzer (Drew Scientific, Miami Lakes, FL). Blood was diluted 1:16 with Isoton II diluent (Beckman-Coulter, Brea, CA) and 4 drops of Zap-o-globin (Beckman-Coulter) was added. After centrifugation at 3,500 rpm for 3 min, the supernatant was added directly to the Hemavet analyzer sample cup. Samples were examined in duplicate. For validation, several whole blood samples were analyzed and compared to the PCV. The cell volume was 3.6%. The assay was found to be linear under dilution ($r^2 = 0.9989$, Runs test, $p = 0.91$).

2.5.2 Biochemistry

The Ortho Vitros 250XR dry slide chemistry analyzer (Rochester, NY) was used for routine biochemistry analyses. This is inclusive of glucose, sodium, chloride, calcium, phosphorus, cholesterol, triglycerides, uric acid, total protein, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase, alkaline phosphatase, and gamma-glutamyl transferase (GGT). Potassium was not measured due to the collection of blood by cardiocentesis and the likelihood of artifactual elevation. The analyzer was calibrated using manufacturer reagents and subject to recommended quality control analysis. Glutamate dehydrogenase (GLDH) was analyzed using reagents from Randox Laboratories (West Kearneysville, VA) on a Randox Daytona analyzer. This test was calibrated with Randox reagents and subject to recommended quality control analysis.

2.5.3 Electrophoresis

Protein electrophoresis was performed using the Helena SPIFE 3000 system with Split Beta gels (Helena Laboratories, Beaumont, TX). Delimits were determined by the scanning software. The electrophoresis system presented results in the form of percentages; these percentages were multiplied by the total protein concentration of each sample to produce the concentrations for each protein fraction. Helena human normal and abnormal controls were used.

2.5.4 Histological analyses

Tissues were processed into paraffin blocks using routine methods and 5-µm sections were placed on glass slides and then stained with hematoxylin and eosin (HE) for analysis.

2.5.5 Group comparisons

In addition to statistical comparisons between treatment and control groups, blood values were reviewed for each individual turtle. Although both species used in this study are common, published, robust reference intervals for hematological and chemical parameters are lacking, particularly for turtles subjected to similar experimental conditions (e.g., e-tubes); definition of species-specific reference intervals was beyond the scope of the current study. Therefore, median values and quartiles for the control groups were considered in combination with available reported values for red-eared sliders and other aquatic turtles, and general ranges used in the clinical evaluation of reptiles (Crawshaw and Holz, 1996; Campbell, 2006; Chaffin et al., 2008; Giménez et al., 2010). Based on this approach, threshold values were developed to describe the degree of deviation from expected central values in case-by-case evaluations (Tables 1 and 2).

Table 1. Threshold values used to characterize the degree of alteration of hematological and chemistry parameters in red-eared slider turtles (*Trachemys scripta elegans)*

Parameter	Very low	Low	Mid-range low	Mid-range high	High	Very high
PCV (%)	15.00	20.00	23.00	27.00	30.00	35.00
RBC count (10^6/ul)	0.70	1.14	1.30	1.63	1.74	1.90
WBC count (10^9/l)	3.00	4.00	6.00	12.00	15.00	20.00
Heterophils (10^9/l)	1.50	2.00	3.00	4.00	5.00	6.00
Lymphocytes (10^9/l)	1.50	2.00	2.50	4.00	5.00	9.00
Monocytes (10^9/l)	0.15	0.30	0.45	0.59	0.71	1.00
Eosinophils (10^9/l)	0.00	0.00	2.00	4.90	6.92	8.95
Basophils (10^9/l)	NA	0.00	1.00	2.20	3.34	4.48
Total protein (g/dl)	2.50	2.98	3.42	4.68	4.90	6.00
Hemoglobin (g/dl)	6.00	7.00	8.00	9.37	10.71	12.05
Albumin to globulin ratio	0.20	0.26	0.30	0.50	0.69	0.75
Total globulins (g/dl)	2.00	2.25	2.42	3.57	3.83	4.50
Albumin (g/dl)	0.60	0.80	1.00	1.14	1.16	2.00
Pre-albumin (g/dl)	NA	0.00	0.05	0.15	0.20	0.25
Alpha 1 globulins (g/dl)	0.06	0.17	0.29	0.52	0.64	0.76
Alpha 2 globulins (g/dl)	0.33	0.47	0.60	0.87	1.01	1.14
Beta globulins (g/dl)	0.38	0.63	0.87	1.35	1.60	1.84
Gamma globulins (g/dl)	0.23	0.45	0.68	1.12	1.34	1.56
Alkaline phosphatase (u/l)	NA	0.00	400.00	696.16	757.00	1,165.72
Aspartate aminotransferase (u/l)	NA	0.00	100.00	226.46	277.31	328.15
Creatine phosphokinase (u/l)	NA	0.00	150.00	396.85	598.78	800.71
GLDH (u/l)	NA	0.00	2.00	7.00	10.00	21.80
Lactate dehydrogenase (u/l)	NA	0.00	250.00	500.00	1,000.00	5,000.00
Calcium (mg/dl)	3.73	5.18	6.63	9.53	10.98	12.43
Phosphorus (mg/dl)	1.90	2.48	3.06	4.22	4.80	5.38
Sodium (mmol/l)	131.17	133.99	136.80	138.00	140.00	143.00
Chloride (mmol/l)	90.00	92.20	95.00	100.00	105.00	115.00
Glucose (mg/dl)	40.00	55.00	65.00	80.00	100.00	120.00
Lipemia index	0.00	0.00	0.00	0.00	0.00	1.00
Cholesterol (mg/dl)	47.16	62.33	77.51	107.87	123.05	138.23
Triglycerides (mg/dl)	30.00	33.40	60.00	170.94	235.65	300.36
Uric acid (mg/dl)	0.10	0.23	0.36	0.61	0.74	2.00

Table 2. Threshold values used to characterize the degree of alteration of hematological and chemistry parameters in common snapping turtles (*Chelydra serpentina)*

Parameter	Very low	Low	Mid-range low	Mid-range high	High	Very high
PCV (%)	15.00	20.00	23.00	27.00	30.00	35.00
RBC count (10^6/ul)	0.60	0.74	0.84	1.00	1.41	1.70
WBC count (10^9/l)	3.00	4.00	6.00	12.00	15.00	20.00
Heterophils (10^9/l)	1.50	2.00	3.00	5.00	7.00	8.00
Lymphocytes (10^9/l)	1.50	2.00	2.50	4.00	5.00	7.00
Monocytes (10^9/l)	0.15	0.30	0.45	0.58	0.61	1.00
Eosinophils (10^9/l)	0.00	0.00	3.00	6.70	9.49	12.29
Basophils (10^9/l)	NA	0.00	5.00	15.38	21.23	27.08
Total protein (g/dl)	2.50	2.64	2.82	3.88	4.14	6.00
Hemoglobin (g/dl)	7.00	8.00	9.00	10.57	11.95	13.34
Albumin to globulin ratio	< 0.20	0.26	0.30	0.50	0.69	0.75
Total globulins (g/dl)	2.00	2.12	2.28	3.33	3.58	4.37
Albumin (g/dl)	< 0.60	0.80	1.00	1.66	1.91	2.50
Pre-albumin (g/dl)	NA	0.00	0.06	0.16	0.21	0.26
Alpha 1 globulins (g/dl)	< 0.03	0.09	0.14	0.25	0.31	0.37
Alpha 2 globulins (g/dl)	< 0.23	0.59	0.95	1.66	2.02	2.38
Beta globulins (g/dl)	< 0.61	0.76	0.91	1.21	1.36	1.52
Gamma globulins (g/dl)	< 0.04	0.10	0.15	0.26	0.32	0.37
Alkaline phosphatase (u/l)	NA	0.00	150.00	300.76	362.99	425.22
Aspartate aminotransferase (u/l)	NA	0.00	100.00	226.46	277.31	328.15
Creatine phosphokinase (u/l)	NA	0.00	75.00	151.54	211.76	271.99
GLDH (u/l)	NA	0.00	2.00	7.00	10.00	24.55
Lactate dehydrogenase (u/l)	NA	0.00	250.00	500.00	1,000.00	5,000.00
Calcium (mg/dl)	< 5.34	6.09	6.84	8.33	9.08	9.83
Phosphorus (mg/dl)	3.93	4.35	4.77	5.61	6.03	6.46
Sodium (mmol/l)	119.80	121.74	123.68	127.55	129.49	131.43
Chloride (mmol/l)	< 76.00	78.20	82.00	85.00	87.00	95.00
Glucose (mg/dl)	40.00	55.00	65.00	80.00	100.00	120.00
Cholesterol (mg/dl)	< 54.62	64.13	73.64	92.67	102.18	111.69
Triglycerides (mg/dl)	100.00	146.60	200.00	265.37	329.35	393.34
Uric acid (mg/dl)	0.32	0.40	0.49	0.67	0.76	2.00

Using these threshold values and ranges, combinations of results (high, low, and normal values for different analytes) were interpreted for each individual animal using defined criteria to characterize patterns of hematological and chemistry results among groups (Table 3). Based on the observed frequency of hematology and chemistry abnormalities in individual turtles, the diagnoses of anemia, dehydration, or possible GI loss or malabsorption were chosen (Table 3). The rationale for selection criteria to establish each diagnosis was based on common patterns of blood work abnormalities in reptiles (Campbell, 2006). Since the interpretation of GI protein loss or malabsorption can be confounded by inflammation and dehydration, low albumin was combined with low globulins to make that diagnosis. The proportions of turtles with anemia, dehydration, or possible GI dysfunction or malabsorption in control and treatment groups were compared by the use of contingency tables and the Fisher exact test (Analyse-it® software). Gross and available histological observations were then considered to investigate and explain potential mechanisms responsible for these results.

Table 3. Criteria used for interpretation of patterns of hematological and blood chemistry results

Interpretation	Criteria
Anemia	PCV below low threshold value *OR* evidence of dehydration *and* PCV below lower mid-range threshold value
Dehydration	Sodium *and* uric acid *or* total protein above high threshold values *OR* sodium *and* chloride above higher mid-range threshold value *OR one or more of the following*: Cl, albumin, PCV above high threshold values
Possible GI protein loss or malabsorption	Albumin *and* total globulins below low threshold values *OR* dehydration *and* albumin *and* total globulins below lower mid-range threshold value

2.6 Bile Analyses for Metabolites of Polycyclic Aromatic Compounds (PACs)

Bile samples collected from 47 red-eared sliders and 52 snapping turtles from the three treatment groups were shipped on dry ice to the Northwest Fisheries Science Center (Seattle, WA) and stored at -20°C until analyses. Turtle bile samples were analyzed using a high-performance liquid chromatography/fluorescence (HPLC-F) method described in Krahn et al. (1984). Briefly, bile was injected directly onto a Waters HPLC-F system equipped with a C-18 reverse-phase column (Phenomenex Synergi Hydro). The fluorescent PAC metabolites were eluted with a linear gradient from 100% water (containing a trace amount of acetic acid) to 100% methanol at a flow of 1.0 mL/min. Chromatograms were recorded at the following wavelength pairs:
(1) 292/335 nm where many 2–3 benzene ring aromatic compounds (e.g., naphthalene) fluoresce,
(2) 260/380 nm where several 3–4 ring compounds (e.g., phenanthrene) fluoresce, and
(3) 380/430 nm where 4–5 ring compounds (e.g., benzo[a]pyrene) fluoresce. Peaks eluting after

9 min were integrated and the areas of these peaks were summed. The concentrations of fluorescent PACs in the bile samples of the turtles were determined using naphthalene (NPH), phenanthrene (PHN), or benzo[a]pyrene (BaP) as external standards and converting the fluorescence response of bile to phenanthrene (ng PHN equivalents g^{-1} bile), naphthalene (ng NPH equivalents g^{-1} bile), or benzo[a]pyrene (ng BaP equivalents g^{-1} bile) equivalents. In addition, protein analysis as described in da Silva et al. (2006) was completed for all bile samples as previous laboratory contaminant exposure studies on fish have shown that normalization of biliary PAC metabolite concentrations to protein values can help account for variation in metabolite levels based on feeding status (Collier and Varanasi, 1991). To ensure that the HPLC-F system was in proper operating condition, a NPH/PHN/BaP calibration standard was analyzed numerous times ($n \geq 5$) until a relative standard deviation (SD) < 15% was obtained for each PAC. As part of the laboratory quality assurance plan (Sloan et al., 2006), a method blank and a fish bile control sample (bile of Atlantic salmon exposed to 25 µg/mL of Monterey crude oil for 48 hours)] were analyzed with each batch of turtle bile samples. In addition, an aliquot of a harbor seal bile sample was also analyzed during the sample sequence as part of the quality assurance plan.

2.7 Biological Endpoints of Oxidative Stress and DNA Damage

The extent of DNA damage in blood and liver cells was assessed using the COMET assay, which is a general, non-specific marker of the induction of DNA strand breaks (Mitchelmore and Chipman, 1998; Mitchelmore et al., 1998). Detailed methods are described in Appendix A. Briefly, levels of total glutathione (tGSH), reduced glutathione (GSHr), and oxidized glutathione (GSSG) were assessed in blood and liver cells using commercially available assays (GT40 from Oxford Biomedical Research, Oxford, MI), as per manufacturer instructions (see Appendix A), with data expressed as µM in terms of WW and mg protein. Total antioxidant levels in plasma and liver cells were quantified using the TA02 total antioxidant power kit from Oxford Biomedical Research (Oxford, MI) as per manufacturer instructions (see Appendix A), and the data are expressed in terms of Trolox, copper reducing, and uric acid equivalents (TEs, CREs, and UAEs, respectively). Levels of lipid peroxidation in whole blood were determined using the thiobarbituric acid reactive substances (TBARS) colorimetric assay kit (FR40) from Oxford Biomedical Research (Oxford, MI), modified for the FR45 fluorescent plate method (see Appendix A), and TBARS assay values are reported in malondialdehyde (MDA) equivalents. The extent of lipid peroxidation in liver tissue was quantified using the FR22 assay kit from Oxford Biomedical Research (Oxford, MI) per manufacturer instructions (see Appendix A). All of the oxidative stress assays are normalized to either mg protein and/or WW of the tissue. To determine protein levels in liver, cell lysates were quantified using the Micro bicinchoninic acid (BCA) Protein Assay Kit (Pierce). Briefly, working reagents and a bovine serum albumin (BSA) standard curve were prepared as outlined in the manufacturer protocol (see Appendix A).

Baseline [i.e., pre-ACTH blood drawn before ACTH injection (in n = 10 turtles) or pre-necropsy (for the remaining n = 5 turtles that are not ACTH challenged)] levels of corticosterone and levels following ACTH stimulation were assessed in turtle plasma using a commercially available corticosterone RIA assay kit (Cornell University).

2.8 Statistical Analyses

Statistical analyses for bile metabolite concentration differences used non-parametric Wilcoxon/Kruskal-Wallis (rank sums) tests. Analysis of variance (ANOVA) and the Tukey-Kramer honestly significant difference (HSD) test (Zar, 1999) were used to compare protein concentrations in bile among the three treatment groups. Protein values were $\log_{10}$-transformed to conform more closely to a normal distribution. For the oxidative stress and DNA damage endpoints, differences between treatments were assessed using one-way ANOVA or Kruskal-Wallis tests. After fitting measured values with one-way ANOVA, the normality of residuals was assessed using the Shapiro-Wilk's test, and if the assumption of normality was rejected ($p < 0.05$), the ANOVA model was estimated again after log-transformation of the measured values. If the assumption of normality remained unmet using transformed values, a Kruskal-Wallis test was applied. In cases where ANOVA was used and an F-test for the main effect of treatments was statistically significant ($p < 0.05$), post-hoc pairwise contrasts between treatment groups were estimated and reported with 95% simultaneous confidence intervals using Tukey's HSD method. All statistical tests were performed using TIBCO Spotfire S-PLUS v8.2 (TIBCO Software Inc.).

3. Results

No turtles of either species died during the study. Some individuals were excluded from data analyses, primarily because they either pulled out their esophagostomy tubes or the tubes were found to be outside of the stomach during necropsy. Overall, 5 control, 6 low-dose, and 3 high-dose turtles were removed from the red-eared slider experiment (total n = 14); therefore, the remaining total numbers in each treatment group were n = 11, n = 11, and n = 13 for the control, low-, and high-dose groups, respectively (of which n = 6, n = 7, and n = 9 underwent the ACTH challenge). Among snapping turtles, 4 control, 3 low-dose, and 3 high-dose turtles were removed (total n = 10), leaving the remaining total numbers of turtles in each treatment group of n = 14, n =14, and n = 15 for the control, low-dose, and high-dose groups (of which n = 8, n = 9, and n = 9 underwent the ACTH challenge). It should be noted that for some of the biological endpoints, a lower total number of samples may have been analyzed due to specific problems with each test (e.g., limited blood volumes, non-quality assurance/quality control compliant co-efficient of variation ranges for replicate assays).

At the end of the study there were no significant differences between treatment groups in either species for weight, carapace length (CL), or condition factor. Likewise, the HSI, the adrenal size index, and the Fulton's condition factor did not differ among treatments for either species (Tables 4 and 5).

Table 4. Mean WW, CL, HSI, condition factor, and adrenal indices for red-eared sliders at necropsy (means ± 1 SD)

Treatment	WW (g)	SCL (mm)	HSI	Condition factor	Adrenal index (WW)	Adrenal index (CL)
0	359.6 ± 54.0	128.5 ± 6.3	0.062 ± 0.013	0.169 ± 0.009	0.152 ± 0.025	0.418 ± 0.053
100	370.2 ± 55.2	126.9 ± 9.5	0.060 ± 0.008	0.183 ± 0.022	0.167 ± 0.016	0.481 ± 0.048
1,000	412.3 ± 102.1	132.9 ± 10.4	0.059 ± 0.011	0.173 ± 0.019	0.149 ± 0.033	0.452 ± 0.093
p-value	0.209	0.254	0.800	0.197	0.384	0.334

HSI = liver WW/carcass WW; Condition factor = $WW/(CL)^3$ x 1,000; Adrenal index (WW) = adrenal area/carcass WW; Adrenal index (CL) = adrenal area/CL.

Table 5. Mean WW, CL, HSI, condition factor, and adrenal indices for snapping turtles at necropsy (means ± 1 SD)

Treatment	WW (g)	CL (mm)	HSI	Condition factor	Adrenal index (WW)	Adrenal index (CL)
0	270.2 ± 36.8	94.1 ± 6.7	0.032 ± 0.003	0.324 ± 0.030	0.226 ± 0.051	0.643 ± 0.143
100	275.8 ± 26.9	94.1 ± 4.4	0.030 ± 0.010	0.330 ± 0.029	0.211 ± 0.046	0.617 ± 0.116
1,000	273.8 ± 44.5	94.7 ± 6.6	0.032 ± 0.006	0.322 ± 0.026	0.226 ± 0.089	0.644 ± 0.240
p-value	0.889	0.964	0.800	0.580	0.523	0.514

HSI = liver WW/carcass WW; Condition factor = $WW/(CL)^3$ x 1,000; Adrenal index (WW) = adrenal area/carcass WW; Adrenal index (CL) = adrenal area/CL.

3.1 Bile Analyses

For both species, PAC bile metabolite concentrations (WW or protein content) exhibited a significant dose-dependent increase among treatments ($p < 0.0001$). Oil-exposed turtles had mean metabolite concentrations that were generally one to two orders of magnitude higher than those measured in the bile of control animals (Figure 1).

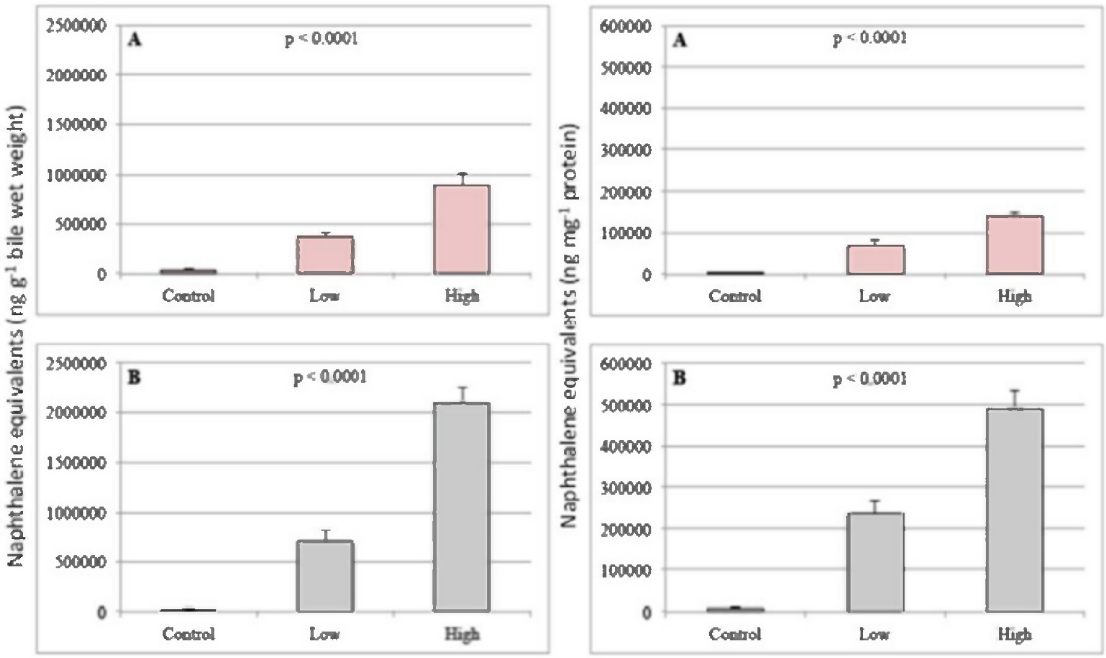

Figure 1. Mean (± SE) concentrations of naphthalene equivalents (ng g^{-1} bile WW on the left and ng mg^{-1} protein on the right) measured in bile of control, low-, and high-oil dosed red-eared sliders (A) and snapping turtles (B). Analyses of phenanthrene and benzo[a]pyrene equivalents showed similar dose-dependent responses.

ANOVA and the Tukey-Kramer HSD test demonstrated that the mean (± SE) biliary protein concentrations of control, low-, and high-oil dosed red-eared sliders were not significantly different (ANOVA $p = 0.3751$) at the $p = 0.05$ level. In contrast, biliary protein concentrations of high-oil dosed snapping turtles were significantly higher (ANOVA $p = 0.0042$) than those determined in snapping turtle controls and low-oil dosed animals. No other differences in protein mean values were found.

3.2 Veterinary Assessment (Blood Chemistry, Hematology, and Histology)

As described earlier, data from 14 red-eared sliders and 10 snapping turtles were excluded due to early necropsies or evidence of confounding conditions that likely were unrelated to oil exposure (e.g., problems with the esophagostomy tube) or irregularity in dosing regimen. Additional turtles were also excluded from the blood data for other reasons (e.g., non-oil-related pneumonia,

lack of significant blood volume). The total numbers of red-eared sliders and snapping turtles included in the analyses in control, low, and high treatment groups are shown in Table 6.

Table 6. Diagnoses among treatment and control groups based on evaluation of hematology and blood chemistry data by individual

Group	Anemia	Dehydration	Possible GI protein loss or malabsorption	Dehydration and possible GI protein loss or malabsorption
RES-control	1/11	8/11	0/11	0/11
RES-low	0/11	11/11	0/11	0/11
RES-high	1/13	9/13	1/13	1/13
SNAP-control	2/13	2/13[a]	2/13[a]	1/13
SNAP-low	0/13	7/13	3/13	3/13
SNAP-high	0/15	11/15[a]	9/15[a]	6/13

RES: red-eared sliders, SNAP = common snapping turtles.

a. Significant differences found between these groups (Fisher exact test $p < 0.05$).

The numbers of turtles affected by anemia, dehydration, and possible GI protein loss or malabsorption are summarized in Table 6 with reference to significant differences between treatment and control groups. Significant differences in various analytes between treatment and control groups are listed in Table 7 for red-eared sliders and Table 8 for common snapping turtles. All turtles in both treatment and control groups had inflammation as identified by electrophoretogram interpretation, and many also by leukogram. Given the presence of inflammation in all study animals, the diagnosis of inflammation was excluded as a toxicity endpoint for comparison among groups. Blood film evaluation revealed neither morphologic abnormalities in blood cells indicative of toxicity or oxidative damage, nor consistent hematologic abnormalities.

Table 7. Significant differences of analytes between treatment and control groups in red-eared sliders

Parameter	Pair-wise comparison with significant contrast
Heterophils (10^9/l)	Low dose-high dose
Hemoglobin (g/dl)	Low dose-high dose
Chloride (mmol/l)	Control-low dose AND low dose-high dose
Phosphorus (mg/dl)	Low dose-high dose
Uric acid (mg/dl)	Control-high dose

Table 8. Significant differences of analytes between treatment and control groups in common snapping turtles

Parameter	Pair-wise comparison with significant contrast
WBC count (10^9/l)	Low dose-high dose
Lymphocytes (10^9/l)	Control-low dose
Eosinophils (10^9/l)	Control-high dose AND low dose-high dose
RBC count (10^6/ul)	Control-low dose AND control-high dose
Pre-albumin (g/dl)	Control-low dose AND low dose-high dose
GGT (u/l)	Control-high dose AND low dose-high dose
Sodium (mmol/l)	Control-high dose
Phosphorus (mg/dl)	Control-high dose AND low dose-high dose
Triglycerides (mg/dl)	Control-low dose AND control-high dose

During gross necropsy, oil or petroleum odor was detected in the gastroenteric contents of all red-eared sliders in the high-dose group, but was not evident in any turtles from the low-dose group. One slider in the high-dose group had edema of the colon wall in addition to the presence of oil. All red-eared sliders in all groups had exudate formation around the e-tube and hepatic lipid accumulation. Additional gross observations included mealworms in the stomach and/or intestines (15/34), mild abrasions on legs or the head associated with the e-tube attachment (6/34), and vitellogenesis (7/34). Histopathological findings for a subset of red-eared sliders (11/34) in the low- and high-dose groups are summarized in Table 9. The most apparent difference among the groups was the diagnosis of moderate subacute gastritis or gastroenteritis in the high-dose group. Turtles in all groups exhibited vacuolar change in the liver compatible with lipid accumulation, as noted grossly.

Table 9. Summary of histopathologic findings in a subset of red-eared sliders

Diagnosis	Hepatic vacuolation	Mild GI inflammation	Moderate GI inflammation	Mild pneumonia	Mild inflammation in various other tissues	Skin mites
Number of turtles	C 5/5	C 3/5	C 1/5	C 2/5	C 5/5	C 0/5
	H 6/6	H 0/6	H 5/6	H 1/6	H 5/6	H 1/6

C = control group, H = high-dose group.

Gross observations in snapping turtles were similar to those in red-eared sliders. Oil or petroleum odor was noted in the GI contents of 13/15 snapping turtles in the high-dose group and none in the low-dose group. Various amounts of exudate associated with e-tube and hepatic lipid accumulation were found in all animals in all three groups. Mealworms were found in the stomach and/or intestines of 22/40 turtles. Histopathological findings of a subset of common

snapping turtles (12/40) from the low- and high-dose groups are summarized in Table 10. The gastritis and enteritis noted in the red-eared sliders was not evident in the snapping turtles. Three turtles in the high-dose group had mild, focal hepatitis. Individuals in both treatment and control groups had focal pulmonary interstitial infiltrates.

Table 10. Summary of histopathologic findings in a subset of common snapping turtles

Diagnosis	Hepatic vacuolation	Mild GI inflammation	Mild focal hepatitis	Focal interstitial pneumonia	Mild inflammation in various other tissues	Skin mites
Number of turtles	C 6/6	C 0/6	C 0/6	C 1/6	C 0/6	C 3/6
	H 6/6	H 0/6	H 3/6	H 2/6	H 0/6	H 3/6

C = control group, H = high-dose group.

3.3 Oxidative Stress and Damage

3.3.1 Total antioxidant levels in blood and liver

In red-eared sliders, no significant differences were observed in blood levels of total antioxidants between treatment groups (Table 11). Higher and significant differences ($p < 0.001$) in total antioxidant levels in red-eared slider liver tissue were observed between control and treatment groups, with higher values in the treatment groups (Figure 2; Table 12). These significantly higher mean levels of CREs were observed in both the low- and high-dose treatment groups compared with the control group. These trends were also significant when the results were calculated in terms of TEs and UAEs.

Table 11. Mean levels of total antioxidants in the blood plasma of red-eared sliders and snapping turtles. There were no significant differences among treatments for any antioxidant endpoint.

		Blood plasma		
Species	Endpoint	Control	Low dose	High dose
Red-eared slider	TEs (µM)	0.233 ± 0.052	0.222 ± 0.036	0.235 ± 0.034
	CREs (µM)	510.374 ± 114.428	486.256 ± 79.980	514.422 ± 74.876
	UAEs (µM)	0.175 ± 0.039	0.167 ± 0.027	0.176 ± 0.025
Snapping turtle	TEs (µM)	0.291 ± 0.084	0.243 ± 0.054	0.224 ± 0.061
	CREs (µM)	637.483 ± 182.855	532.783 ± 117.455	490.718 ± 133.243
	UAEs (µM)	0.219 ± 0.063	0.183 ± 0.040	0.169 ± 0.046

Results are presented as means ± SD.

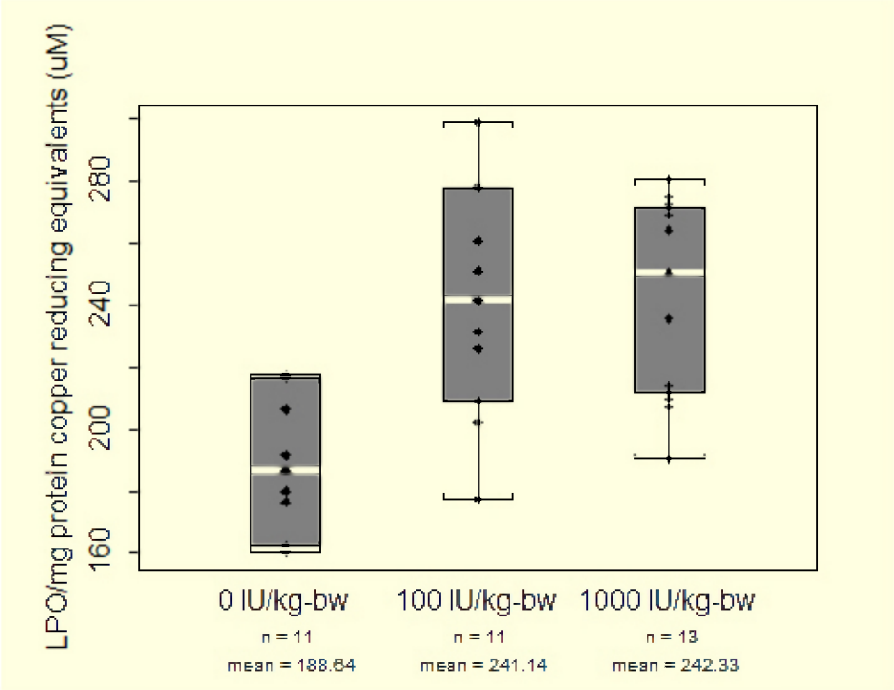

Figure 2. Total antioxidants in liver tissue presented per mg protein in terms of CREs. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

Similar to red-eared sliders, antioxidants in snapping turtle blood showed no significant differences among treatment groups (Table 11), but snapping turtle liver tissue showed increased total antioxidant levels with increasing dose ($p = 0.012$). Mean control CREs, TEs, and UAEs were significantly different from the low- and high-dose treatment groups when presented as µM. However, when indexed to mg protein, these endpoints did not differ among treatment groups ($p = 0.273$).

Table 12. Mean levels of total antioxidants in the liver cells of red-eared sliders and snapping turtles

		Liver tissue		
Species	**Endpoint**	**Control**	**Low dose**	**High dose**
Red-eared slider	TEs (µM)	0.606 ± 0.137	0.686 ± 0.115	0.734 ± 0.124
	CREs (µM)	1,327.278 ± 251.403	1,503.524 ± 251.403	1,607.671 ± 272.770
	UAEs (µM)	0.456 ± 0.103	0.516 ± 0.086	0.552 ± 0.094
	TEs/mg protein (µM)	0.086 ± 0.010	0.110 ± 0.017#	0.111 ± 0.014*
	CREs/mg protein (µM)	188.636 ± 22.852	241.143 ± 36.656#	242.331 ± 31.995*
	UAEs/mg protein (µM)	0.065 ± 0.008	0.083 ± 0.013#	0.083 ± 0.011*
Snapping turtle	TEs (µM)	0.824 ± 0.099	0.871 ± 0.097	0.951 ± 0.119*
	CREs (µM)	1,804.225 ± 216.374	1,905.943 ± 212.269	2,080.708 ± 260.177*
	UAEs (µM)	0.620 ± 0.074	0.655 ± 0.073	0.715 ± 0.089*
	TEs/mg protein (µM)	0.094 ± 0.009	0.100 ± 0.010	0.101 ± 0.013
	CREs/mg protein (µM)	205.886 ± 21.604	218.351 ± 22.245	221.126 ± 29.049
	UAEs/mg protein (µM)	0.071 ± 0.007	0.075 ± 0.007	0.076 ± 0.009

Results are presented as means ± SD. Results are presented as means ± SD. Significant differences (ANOVA and Tukey post-hoc test using $p < 0.05$), *$P < 0.05$ control vs. high dose, #$P < 0.05$ control vs. low dose, and ^$P < 0.05$ low vs. high dose.

3.3.2 Glutathione levels in blood and liver

Levels of total, oxidized, and reduced GSH were not significantly different between control and treatment groups in the blood of both red-eared sliders and snapping turtles (Table 13). Of note is that in the red-eared slider blood samples, levels of GSH (µM) did appear to decrease as the dose of treatment increased, although this was not significant given the large variability in the individuals. The only significant differences among treatments were found in liver tissue of red-eared sliders (Table 14). Levels of GSSG (as µM; $p = 0.001$ and as per mg protein; $p < 0.001$) increased with oil dose (Figure 3). However, in post-hoc tests, given the large variability in values in the low-dose group, only the high-dose treatment group was statistically significant from the control group ($p < 0.001$). In the liver tissue, mean GSH (µM) levels also showed a significant increase with dose (Figure 4), although when presented in terms of mg protein in the sample, these results were not significant ($p = 0.084$). Similarly, GSHr levels in red-eared slider liver tissue also showed a significant ($p = 0.048$) increase with increasing dose (see Table 14). However, when incorporating mg protein in liver samples, these results were not significant ($p = 0.086$). In the snapping turtle groups, no GSH endpoints in the treatment groups were significantly different from the control groups for either blood or liver tissue (Tables 13 and 14).

Table 13. Mean levels of total, reduced, and oxidized GSH in blood in both species

		Blood plasma		
Species	**Endpoint**	**Control**	**Low dose**	**High dose**
Red-eared slider	GSH (µM)	1,368 ± 268	1,256 ± 247	1175 ± 268
	GSHR (µM)	1,360 ± 268	1,247 ± 248	1166 ± 267
	GSSG (µM)	4.23 ± 0.95	4.49 ± 1.49	4.55 ± 3.14
	GSH:GSSG ratio	337 ± 103	309 ± 116	398 ± 320
Snapping turtle	GSH (µM)	1,701 ± 232	1,572 ± 274	1657 ± 287
	GSHR (µM)	1,687 ± 226	1,560 ± 275	1644 ± 287
	GSSG (µM)	7.21 ± 7.45	6.03 ± 2.00	6.52 ± 1.8
	GSH:GSSG ratio	322 ± 130	291 ± 119	278 ± 130

Results are presented as means ± SD.

Table 14. Mean levels of total, reduced, and oxidized GSH in liver cells in both species

		Liver tissue		
Species	**Endpoint**	**Control**	**Low dose**	**High dose**
Red-eared slider	GSH (µM)	1,419 ± 723	1,760 ± 605	2,169 ± 955*
	GSHR (µM)	1,412 ± 723	1,750 ± 605	2,157 ± 953*
	GSSG (µM)	3.81 ± 0.68	4.98 ± 1.69	6.10 ± 1.40*
	GSH:GSSG ratio	395 ± 263	550 ± 803	352 ± 113
	GSH/mg protein	142 ± 67.3	170 ± 57.7	205 ± 73.0
	GSHR/mg protein	141 ± 67.3	169 ± 57.6	203 ± 72.9
	GSSG/mg protein	0.339 ± 0.064	0.432 ± 0.155	0.514 ± 0.104*
	GSH:GSSG ratio/mg protein	438 ± 255	637 ± 975	396 ± 120
Snapping turtle	GSH (µM)	1,381 ± 297	1,298 ± 310	1,507 ± 501
	GSHR (µM)	1,365 ± 296	1,283 ± 310	1,490 ± 500
	GSSG (µM)	7.76 ± 2.24	7.69 ± 2.48	8.44 ± 1.77
	GSH:GSSG ratio	194 ± 82.6	199 ± 133	182 ± 68.3
	GSH/mg protein	113 ± 26.0	108 ± 29.6	109 ± 33.9
	GSHR/mg protein	112 ± 25.8	107 ± 29.4	108 ± 33.8
	GSSG/mg protein	0.550 ± 0.185	0.577 ± 0.212	0.580 ± 0.164
	GSH:GSSG ratio/mg protein	225 ± 78.7	217 ± 122	197 ± 75.9

Results are presented as means ± SD. Significant differences (ANOVA and Tukey post-hoc test using $p < 0.05$), *$P < 0.05$ control vs. high dose, #$P < 0.05$ control vs. low dose.

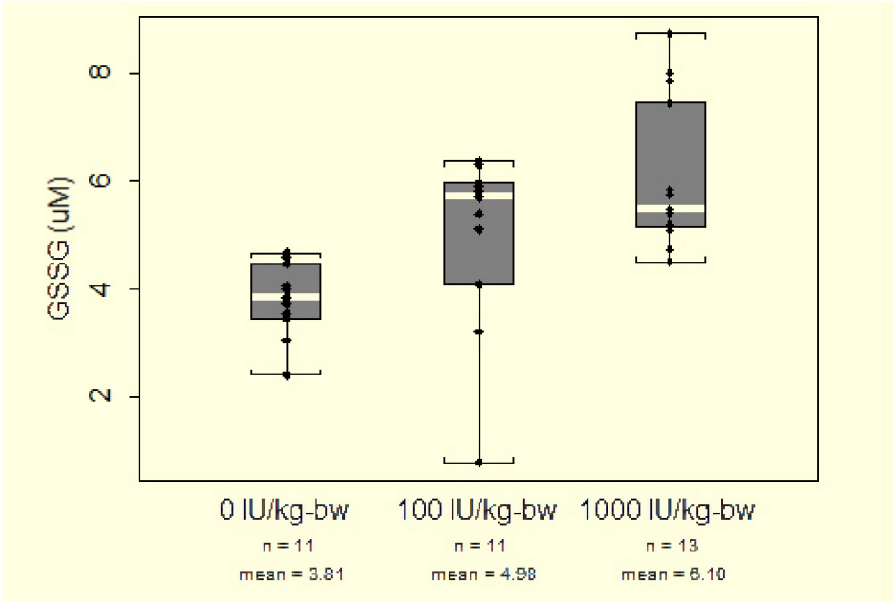

Figure 3. Levels of GSSG in red-eared slider liver tissue expressed as µM GSH increased significantly between the control and high-dose treatment groups. Levels of GSSG per mg protein also showed significant dose-dependent increase ($p < 0.001$). Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

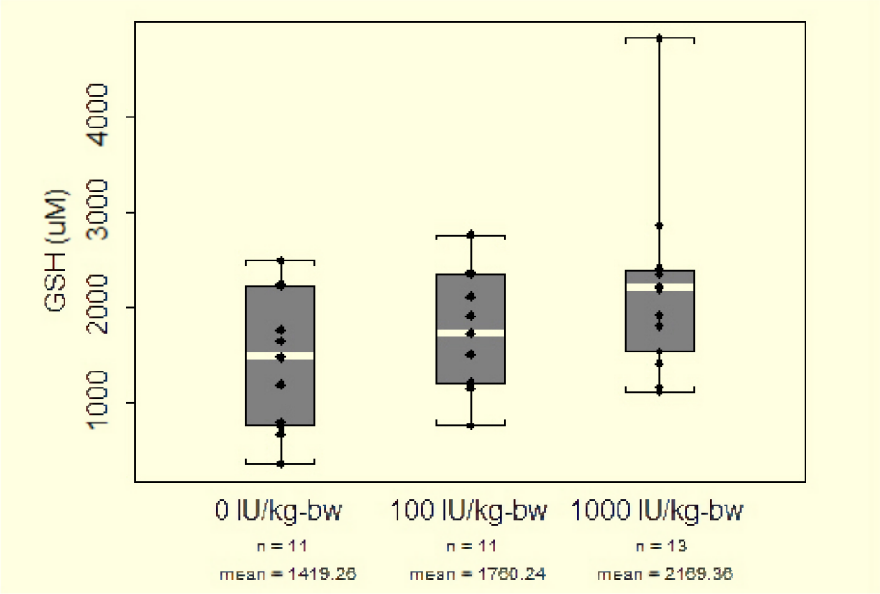

Figure 4. Levels of tGSH in red-eared slider liver tissue (µM) increased significantly between the control and high dose treatment groups. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

3.3.3 Lipid peroxidation in blood and liver cells

No significant differences among treatment groups were observed in the blood plasma ($p = 0.862$) or liver tissue ($p = 0.497$) in red-eared sliders (Table 15). Of note is that MDA concentrations (nm) in plasma increased with dose increases (i.e., 241, 336, and 404 for control, low-, and high-dose groups, respectively), although variation between individuals was very high in the control and high-dose groups. Plasma levels of MDA were 17 times higher than levels reported in red-eared slider liver tissue.

In snapping turtles, levels of lipid peroxidation were significantly different [MDA (nm)] in both plasma ($p = 0.001$) and liver tissue ($p = 0.008$) with plasma MDA concentrations increasing with dose (Table 15 and Figure 5). However, in snapping turtle liver tissue, MDA concentrations (nm) showed a different trend from plasma. Liver tissue MDA showed a decrease from the control to the low-dose group and an increase from the low- to the high-dose group (see Table 15). When incorporating mg protein in liver samples, the results are not significant ($p = 0.82$).

Table 15. Mean level of lipid peroxidation in blood and liver tissue of both turtle species

Species	Endpoint	Control	Low dose	High dose
			Blood plasma	
Red-eared slider	MDA (nm)	297 ± 108	265 ± 27.4	278 ± 59.3
Snapping turtle	MDA (nm)	241 ± 13.3	336 ± 90.4#	404 ± 65.8*
			Liver tissue	
Red-eared slider	MDA (nm)	12.1 ± 4.85	12.0 ± 2.73	27.7 ± 38.6
	MDA/mg protein	1.45 ± 0.57	1.66 ± 0.68	3.28 ± 4.00
Snapping turtle	MDA (nm)	14.1 ± 3.24	12.9 ± 5.51^	20.4 ± 8.87*
	MDA/mg protein	1.14 ± 0.42	1.06 ± 0.44^	1.52 ± 0.67*

Results are presented as means ± SD. Significant differences (ANOVA and Tukey post-hoc test using $p < 0.05$), *$P < 0.05$ control vs. high dose, #$P < 0.05$ control vs. low dose., ^$P < 0.05$ low vs. high dose.

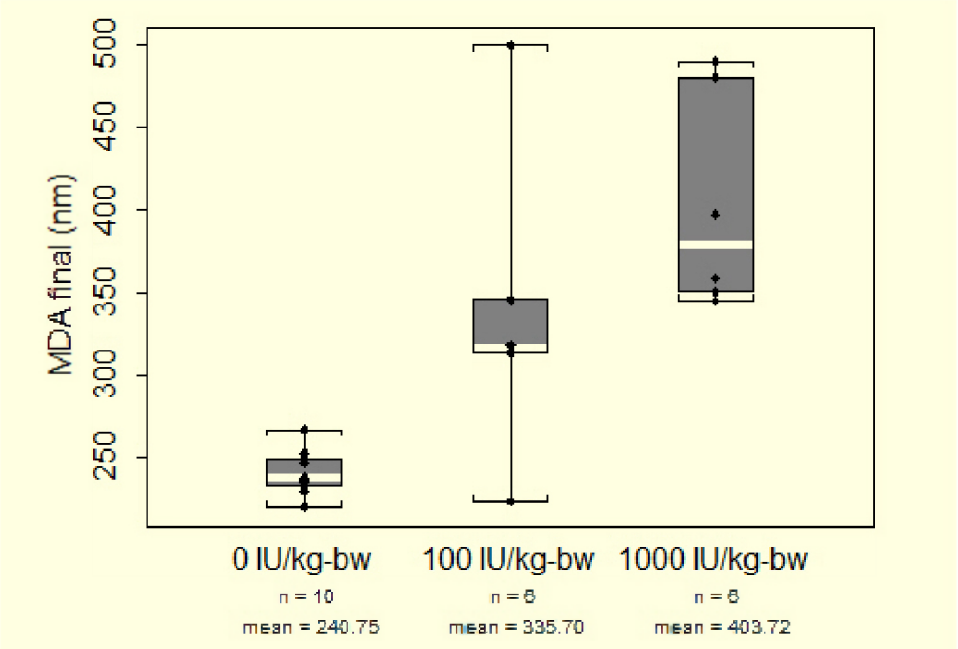

Figure 5. Lipid peroxidation increased significantly in snapping turtle plasma with increasing oil dosage. Both treatment groups were significantly different than the control group, but not different from each other. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

3.4 DNA Damage (COMET Assay)

Very low levels of DNA damage were observed in blood and liver cells in both red-eared sliders and snapping turtles in all of the treatment groups (Tables 16 and 17). Levels of DNA damage in terms of the percentage of DNA in the tail all averaged less than 6% and 9% in blood and liver cells in both species, with all individual levels below 12% in the red-eared sliders and 12% in the snapping turtles. No statistically significant differences in levels of DNA damage in liver cells were observed in red-eared sliders using all three endpoints (tail length, olive tail moment, and percent tail DNA; Table 17). The only statistically significant differences in percent tail DNA were observed between control and high-dose treatment groups in blood cells of red-eared sliders ($p = 0.003$) (Figure 6). Olive tail moment showed a similar increasing trend with increasing oil dose, but these results were not significant ($p = 0.112$).

In snapping turtles, there was a statistically significant difference in DNA damage when expressed as comet tail length in blood among all treatment groups ($p < 0.001$). However, the highest levels of DNA damage (as tail length) were observed in the low-dose group (Figure 7). No significant differences were observed in snapping turtles with either cell type when DNA damage was expressed as tail % DNA, although olive moment was significantly lower ($p = 0.003$) in the low-dose treatment group than the control and high dose treatment groups (Table 17).

Table 16. Levels of DNA damage in blood cells of red-eared sliders and snapping turtles

		Blood		
Species	**Endpoint**	**Control**	**Low dose**	**High dose**
Red-eared slider	Tail DNA (%)	5.007 ± 0.558	4.932 ± 0.547	5.211 ± 0.803
	Tail length (µm)	2.525 ± 0.179	2.723 ± 0.445	3.015 ± 0.303*
	Olive moment	0.187 ± 0.021	0.206 ± 0.028	0.214 ± 0.038
Snapping turtle	Tail DNA (%)	5.319 ± 0.784	5.0714 ± 0.613	5.041 ± 1.567
	Tail length (µm)	2.874 ± 0.379	4.516 ± 0.828# ^	3.647 ± 0.946*
	Olive moment	0.219 ± 0.041	0.294 ± 0.054	0.243 ± 0.070

Results are presented as means ± SD. Significant differences (ANOVA and Tukey post-hoc test using $P < 0.05$), *$P < 0.05$ control vs. high dose #$P < 0.05$ control vs. low dose., ^$P < 0.05$ low vs. high dose.

Table 17. Levels of DNA damage in liver cells of red-eared sliders and snapping turtles

		Liver tissue		
Species	**Endpoint**	**Control**	**Low dose**	**High dose**
Red-eared slider	Tail DNA (%)	5.199 ± 1.187	6.485 ± 1.602	5.801 ± 1.555
	Tail length (µm)	3.481 ± 1.252	4.411 ± 1.729	4.176 ± 1.760
	Olive moment	0.204 ± 0.075	0.262 ± 0.083	0.25 ± 0.135
Snapping turtle	Tail DNA (%)	8.494 ± 1.343	7.916 ± 1.218	8.285 ± 1.541
	Tail length (µm)	7.176 ± 1.404	6.127 ± 1.185	7.205 ± 1.376
	Olive moment	0.451 ± 0.131	0.374 ± 0.060#	0.461 ± 0.109

Results are presented as means ± SD. Significant differences (ANOVA and Tukey post-hoc test using $P < 0.05$), #$P < 0.05$ control vs. low dose.

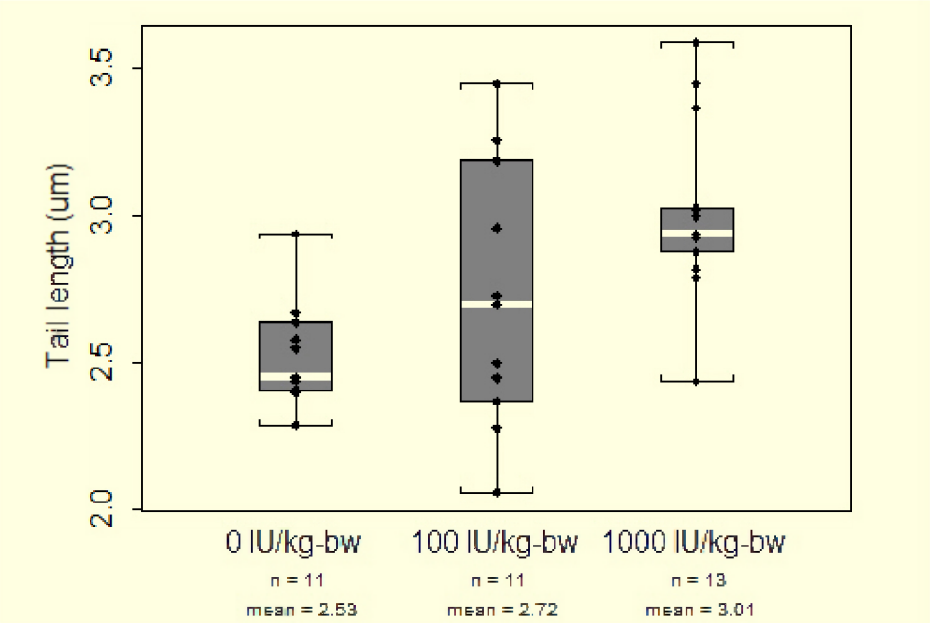

Figure 6. Level of DNA damage in red-eared slider blood cells, presented as tail length (µM) in the COMET assay, increased significantly with oil dose between control and high-dose treatment groups. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

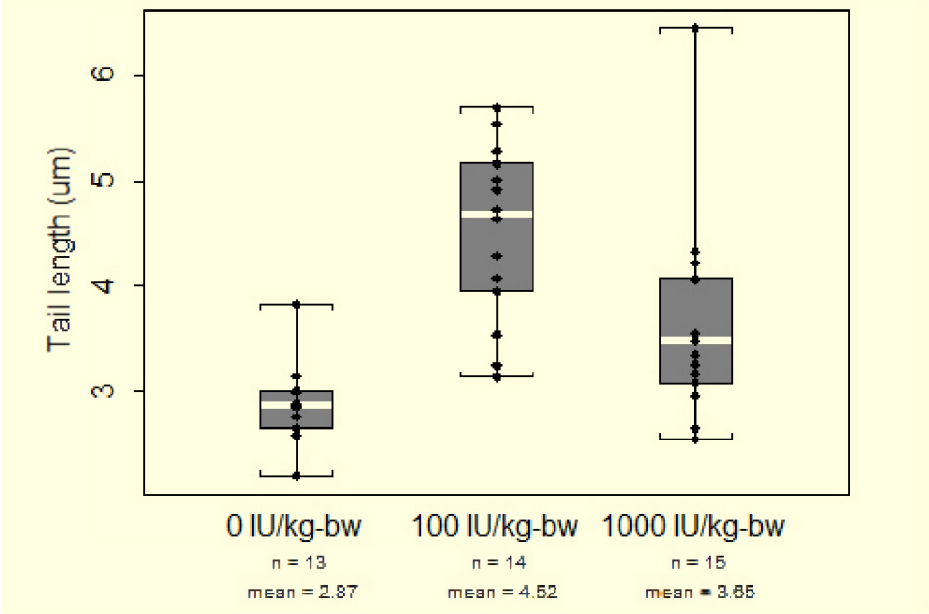

Figure 7. Level of DNA damage in snapping turtle blood cells, presented as tail length (µM) in the COMET assay, varied significantly among all treatment groups. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

3.5 Potential HPA Axis Dysfunction

To investigate the response to stress induction, we calculated the percentage change in levels of corticosterone after ACTH induction vs baseline corticosterone levels. A non-statistically significant decrease in red-eared slider plasma was observed (Figure 8). The lack of statistical significance was probably due to the limited number of turtles and high inter-individual variability. However, it should be noted that two red-eared sliders in the high-dose oil group showed dysfunction of the HPA axis as induction levels were much lower compared to what would be considered a normal response.

In snapping turtles, no statistically significant differences in blood plasma were observed in the percentage change in corticosterone levels following ACTH response between control and treatment groups (Figure 9).

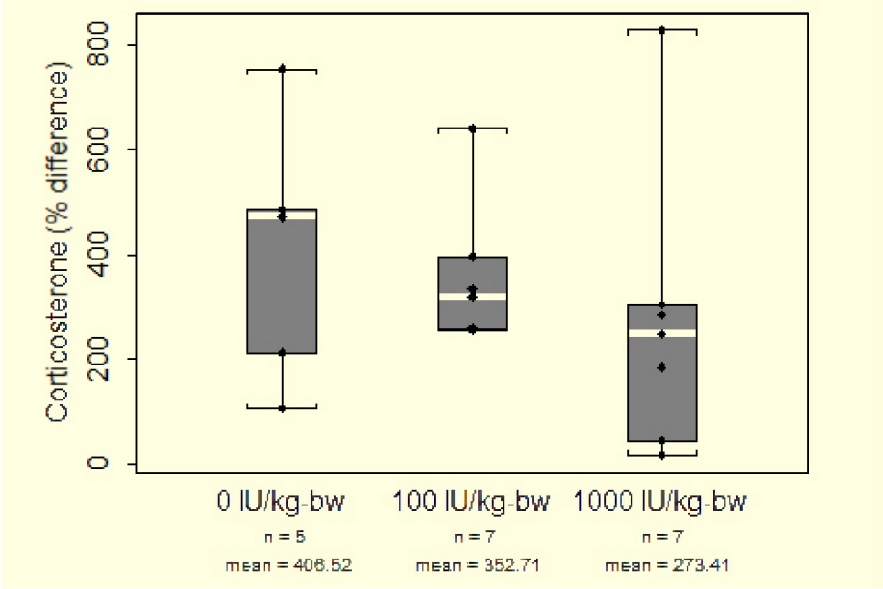

Figure 8. Corticosterone levels in blood plasma of red-eared sliders following ACTH induction. Although among-treatment differences were not statistically significant, two high-dose turtles failed to respond to clinically normal levels. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

4. Discussion and Conclusions

Overall, turtles orally exposed to DWH oil during the surrogate study did not show severe, life-threatening physiological derangements or mortality. Most turtles continued to feed despite having clearly visible oil within the GI tract in the high-dose group. The volume of oil administered in the high-dose group was near the maximum amount that can be given by ingestion without resulting in regurgitation based on pilot phases of this project. Potential effects of esophageal administration of oil on hematology and blood chemistry parameters in the species used in this study were mainly associated with the GI tract and possible related hemodynamic derangements. Anemia and oxidative damage to RBCs, as reported in other species exposed to petroleum, were not observed. Consistent gross and histopathological abnormalities were also not observed, which is consistent with findings in sea turtles oiled during the DWH spill (Stacy, 2012). These results were consistent with other observations that effects from physical fouling are the most readily apparent consequence of oil exposure in sea turtles (Stacy, 2012).

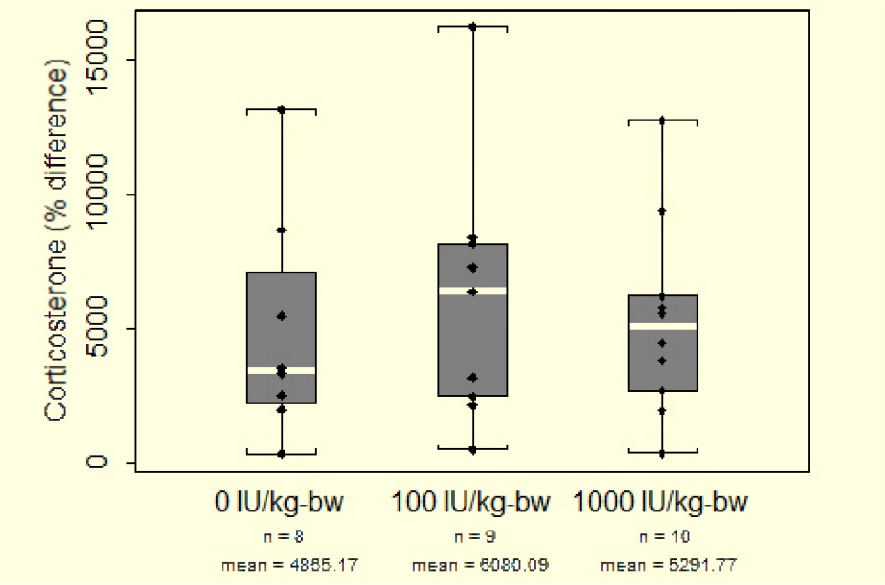

Figure 9. Corticosterone levels in blood plasma of snapping turtles following ACTH induction. Box and whisker plots show median (white bar), interquartile range (grey box), and minimum and maximum values (vertical bars). All data points used in analyses also shown.

4.1 Bile Analysis

There was a clear dose-dependent increase in the levels of fluorescent PAH metabolites and protein concentrations that were measured in bile samples of red-eared sliders and snapping turtles from the three treatment groups (Figure 1). Dose-dependent increases in the levels of biliary PAH metabolites demonstrated exposure, uptake, and metabolism of oil at levels similar or higher to those measured in the limited number of field-collected sea turtles for which bile PAH metabolite data was available (see Ylitalo et al., 2014). For example, field-collected animals contained PHN equivalents (not protein corrected) in Category 2 (n = 1) and Category 4 (n = 4) at 18,000 and 370,000 PHN equivalents/mL bile. In the low-dose surrogate turtles, these levels were 358,000 and 517,000 PHN equivalents/mL bile for red-eared sliders and snapping turtles, respectively.

Higher concentrations of biliary protein in high-oil dosed snapping turtles could indicate that these animals may have had a reduced intake or assimilation of food compared to control or low-oil dosed snapping turtles, as biliary protein has been shown to increase in non-feeding vertebrates such as fish (Collier and Varanasi, 1991). Animals in this study received a daily food bolus as part of the oil dose (or control dose), were also given 10 mealworms per day, and leftover food was noted. Consumption of the mealworms and fecal production did not appear to differ in either species between treatments groups.

4.2 Veterinary Assessment (Blood Chemistry, hematology, and Histology)

Interpretation of some parameters was clearly confounded by inflammation associated with esophagostomy tubes. For example, the diagnosis of systemic inflammation based on electrophoretograms in all study turtles – including control animals – precluded its use as an endpoint of oil toxicity. Meaningful interpretation of significant differences in leukocyte counts among control and treatment groups was similarly confounded. Although inflammation associated with the e-tubes confounded interpretation of some endpoints, the tubes were required to consistently administer the oil dosages necessary to reliably approximate the level of exposure estimated for naturally exposed sea turtles. Furthermore, the tubes provided a relatively low stress and time-efficient route of administration as compared to gavage feeding, which was highly unreliable in the pilot phase.

In addition to inflammation, evidence of dehydration based on the blood chemistry data was frequently observed in this study. The significant variation in proportions of snapping turtles with dehydration among treatments could reflect a treatment-related effect of water consumption, absorption, or loss in this species. However, evidence of dehydration in many of the control sliders cannot be ignored and raises the possibility of some other effect unrelated to oil administration, especially the esophagostomy tubes and associated inflammation.

There was evidence of GI protein loss or malabsorption in the snapping turtles dosed with oil. Ten snapping turtles had evidence of both dehydration and protein loss/malabsorption, nine of which were given oil. Concurrent inflammation and dehydration may have masked a similar effect on the red-eared sliders. Plasma proteins are composed of positive and negative acute phase proteins that demonstrate typical patterns of increase or decrease with inflammation. Dehydration also typically increases plasma proteins; thus, inflammation and dehydration can obscure protein loss or reduced absorption within the GI tract (Stockham and Scott, 2008). Given that there was no concurrent GI inflammation or ulceration observed in the snapping turtles, effects of oil on the digestive system may result from physical or physiological alteration of motility or absorption. The bile protein content data discussed above potentially corroborates the hematological evidence for reduced feeding rate or assimilation of food in the snapping turtles.

Significance of the higher hemoglobin concentration in red-eared sliders in the low-dose group compared to the high-dose group is not supported by concurrent differences in PCV or RBC count. While the majority of high-dose red-eared sliders had hemoglobin within normal range, only two sliders of the high-dose group had mildly decreased hemoglobin and mild anemia, with one turtle having a mildly increased RBC count. Considerations for these findings include pericardial fluid contamination during blood withdrawal, individual variations, or laboratory variance. Contamination of blood with pericardial fluid did not appear to be a problem in this study, but cannot be completely ruled out. Significantly higher RBC counts in control animals compared to both treatment groups in snapping turtles is also not supported by significant differences in PCV or presence of anemia in treatment groups. RBC counts were highly variable in snapping turtles, which may be explained by laboratory and/or individual variations. For example, four snapping turtles with anemia had high (n = 1), high normal (n = 2), or low RBC counts (n = 1). Mild anemia was diagnosed in a small proportion of study animals, including control animals. This observation may be due to pericardial fluid contamination during blood withdrawal, chronic inflammation, or non-pathological inter-individual differences.

The lack of consistency in significant differences in electrolyte, phosphorus, and uric acid concentrations among the treatment and control groups of both species, as well as individual variability within groups, suggests that the observed alterations are due to a combination of factors, such as differences in hydration status, inflammatory response, GI function, food intake, individual and species variation, as well as an effect of relatively low sample size. Red-eared sliders in the low-dose group had higher chloride concentrations compared to the high-dose and control groups, and higher phosphorus compared to the high-dose group. Mean uric acid concentration was higher in the high-dose group compared to the control group. All three analytes can be elevated with dehydration. For example, one high dose red-eared slider had elevated uric acid and concurrently high sodium and chloride concentrations, which is consistent with dehydration. This individual contributed to the overall significantly higher mean uric acid concentration in the high-dose group. Despite the significant differences in phosphorus concentrations between red-eared sliders in the high- and low-dose groups, all were within normal limits. In snapping turtles, hydration status is the most likely explanation for the higher sodium concentration in the high-dose group compared to the control group. The lower phosphorus concentration in the high-dose snapping turtle group is attributable to malabsorption, as previously discussed. Similarly, differences in triglyceride concentrations between the control and treatment groups also may reflect malabsorption in snapping turtles; however, these results cannot be interpreted because concurrent lipemia in some of the snapping turtles appears to have interfered with lipid analysis.

Pre-albumin is a negative acute-phase protein; thus, interpretation of the differences among some of the snapping turtle groups is confounded by the presence of systemic inflammation (Cray et al., 2007). In addition, high-dose snapping turtles had significantly higher GGT than control and low-dose groups, although overall concentrations were comparably low and would not be interpreted clinically as elevated. GGT is an indicator of cholestasis and biliary hyperplasia and

can be increased with some drugs in mammals (Stockham and Scott, 2008), but this enzyme has not been validated for its diagnostic usefulness in reptiles. The observed hepatic lipid accumulation, which is attributed to captive diet and husbandry in this study, could have contributed to enzyme variations among groups in snapping turtles.

Overall, the veterinary assessment documented physiological abnormalities, including evidence of dehydration, decreased digestive function and assimilation of nutrients, and altered immune responses. However, none of these conditions were associated with death, nor with grave prognosis for survival in turtles in this study.

4.3 Oxidative Stress and Damage (Blood, Liver, and DNA)

Evidence of oil-derived increases in oxidative stress and associated damage (to lipids or DNA) were either not apparent or relatively minor in both species in blood and liver tissues. Increased levels of lipid peroxidation and DNA damage are often observed in species exposed to oil and/or PAHs, mainly due to their metabolism to either reactive, carbon-centered electrophilic intermediates or carbon- and/or oxygen-centered free radical species (Mitchelmore et al., 1998). To minimize oxidative damage to cellular components an array of constitutive and up-regulated antioxidant enzyme systems and free-radical scavengers (e.g., GSH, vitamins) are involved. However, it is often difficult to interpret these results as both reductions and elevations can occur depending upon the chemical, dose, and time post-exposure. For example, a loss of GSH in conversion to GSSG, or when conjugated to a PAH metabolite and excreted, may be observed. Similarly, compensatory responses to PAH exposure may show an elevation in levels of GSH compared with controls. GSSG levels are usually increased and hence GSH:GSSG ratios decreased during exposure to PAH metabolites. However, only the red-eared sliders had significant increases in GSSG levels with oil exposure, but only in the liver and without a concurrent decrease in GSH levels. GSH levels in blood also decreased with increasing dose, but this trend was not significant. Evidence of hepatic up-regulation of additional antioxidant species in red-eared sliders and snapping turtles was observed as significantly higher levels in antioxidant species with increasing dose were found in liver tissue, but not in blood. Taken together, oxidative stress or damage endpoints analyzed in this study did not show consistent responses to oil exposure.

4.4 Potential HPA Axis Dysfunction

In contrast to some studies of petroleum toxicity in other vertebrates (e.g., Mohr et al., 2010; Lattin et al., 2014; Schwacke et al., 2014), no evidence of HPA dysfunction was apparent in snapping turtles, nor in most red-eared sliders. However, two red-eared sliders that had received a high dose of oil showed dysfunction of the HPA axis, which regulates stress response and other

vital functions. When all individuals were compared by oil-dose treatment, red-eared sliders that had received an oil dose showed a reduced and dose-dependent stress response; however, this dysfunction was not statistically significant, likely due to the high variation among the limited number of individuals. Furthermore, turtles were dosed with oil for only 14 days, which is a much shorter time period than for other species (e.g., > 60 days in mink; Mohr et al., 2010). Therefore, the effects of experimental oil exposure on red-eared sliders and common snapping turtles did not clearly indicate HPA dysfunction as reported in other vertebrate species; however, given the results in the red-eared sliders, potential HPA dysfunction caused by oil exposure cannot be ruled out.

5. Conclusions

Overall, red-eared sliders and snapping turtles orally exposed to MC 252 oil for 14 days during the surrogate study did not show mortality or severe life-threatening physiological derangements. Turtles were clearly internally exposed to and metabolized oil constituents as evidenced by the dose-dependent increases in bile metabolites. Oil-related alterations in food assimilation, malabsorption, and dehydration, were observed. Additionally, HPA axis function was not significantly affected by oil exposure, but possible effects could not be completely ruled out. Some alterations in antioxidant levels and measures of oxidative stress and DNA damage were consistent with PAH exposures, but were not consistent across endpoints and treatment groups. However, there was an inability to dose surrogate turtles for time periods comparable to other studies where such effects were shown in orally dosed vertebrates. Delayed and longer-term effects of these low-dose oil exposures, as well as exposure via multiple routes, as are relevant to turtle exposure during oil spills, also were not part of the study design due to logistical constraints.

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Appendix. Work Plan for Phase 4 Turtle Surrogate Study

1.1 Phase Four Study: Oral Dosing of Oil in Turtles

For the fourth phase, red-eared sliders and snapping turtles will be dosed orally with oil for 14-days. The first experiment will be carried out with the red-eared sliders and the second experiment with the snapping turtles. The objective of this study is to determine sub-lethal impacts to turtles following exposure to oil. A variety of biological and chemical endpoints will be used to determine exposure to and effects of the oil. Specifically, endpoints will investigate the extent of PAH metabolites, oxidative stress, histological modifications and impacts to hypothalamic-pituitary-adrenal axis (HPA) in addition to blood chemical and biological endpoints. This study will use Slick A and consist of two oil doses and a control group.

1.1.1 Experimental design

For this phase of the study, both red-eared sliders and snapping turtles will be tested in separate experiments. The study will include two treatment groups plus a control group. The control group will be dosed orally with the food slurry bolus followed by the 0.9% sterile saline to account for potential stress related to dosing and handling while the treatment groups will be dosed orally with daily doses of Slick A oil followed by the food slurry bolus and 0.9% sterile saline. Each group will have 16 animals for a total of 48 animals. Six of the turtles in each group will be necropsied at the end of the 14-day period, the other ten will first have a pre-dose blood draw taken (for baseline corticosterone levels and for blood chemical and biological endpoints), followed by a dose of ACTH, after a set-period of time [see Standard Operating Procedure (SOP) #10] they will then undergo necropsy so that a post-dose ACTH blood draw can also be taken.

To dose the turtles orally, feeding e-tubes will be surgically inserted by a trained veterinarian according to SOP #6 at least 4 days before experimental dosing. Each individual turtle will be weighed (see SOP #1) and dosed on a daily basis for the 14-day period unless signs of stress occur or that they remove their e-tube resulting in their early termination from the study. Turtles will be inspected daily for signs of feeding, regurgitation, feces production and any other signs of stress/discomfort including issues related to the e-tube surgical site. At the end of the 14-day study, all turtles will be necropsied and blood/tissue samples taken and stored appropriately or sent directly to the contracting laboratories as outlined in Table 1. Ten of the 16 turtles in each group will undergo ACTH challenge to examine adrenal response to oil dosing. This will include a pre-ACTH blood collection to establish a corticosterone baseline (and to analyze blood chemistry and biological endpoints), injection of ACTH, and final blood draw following the challenge. Necropsies will be performed and tissue samples processed and collected according to SOP #8. Blood samples will be collected according to SOP #3b.

Table 1. Analysis detail, sample containers, preservation techniques, and holding times for each type of sample/analysis/ endpoint to be conducted (priority samples highlighted in bold text). *Samples are priority level 2 and will be stored for potential analysis at a later date.

Analysis	Sample type (minimum volume)	Preparation details	Storage container	Storage conditions	Analysis lab/ sent details
RBC/WBC counts and morphology (1)	60 µL heparinized whole blood	Blood smear; prepare 3 slides at CBL (SOP #2) and 3 slides at Miami	Glass slides in plastic slide box	R.T. in slide box	Send to Miami and N. Stacy (for morphology) at R.T.
RBC/WBC counts and morphology (2); hematocrit (PCV)	100 µL heparinized whole blood	Remove from vacutainer after careful mixing	Labeled plastic 1.5 mL cryovial (internal O-ring)	Keep at 4°C, do not freeze or place direct on ice	Send to Miami and N. Stacy (for morphology) O/N on blue ice
Hemoglobin concentration	100 µL heparinized whole blood (500 µL for validation)	Remove from vacutainer after careful mixing	Labeled plastic 1.5 mL cryovial (internal O-ring)	Keep at 4°C, do not freeze or place direct on ice	Send to Miami O/N on blue ice
PCV	30-40 µL heparinized whole blood	Remove from vacutainer after careful mixing	Microhematocrit capillary tube; see SOP #9	Keep at 4°C do not freeze or place direct on ice; analyze in < 12 h	Analysis at CBL
Blood chemistry panel (based on bird work and reptile panel): see Table 2 for list	320 µL heparinized plasma (for all) except GLDH	Spin whole blood at 2,000 g for 10 min at 10°C, remove supernatant (plasma)	Labeled plastic cryovials with internal O ring (one for 300 µL and one for 100 µL sample)	Freeze on dry ice and store at -70°C	Send to Miami O/N on dry ice
GLDH	100 µL heparinized plasma	Spin whole blood at 2,000 g for 10 min at 10°C, remove supernatant (plasma)	Labeled plastic cryovials with internal O ring	Freeze on dry ice and store at -70°C	Send to Miami O/N on dry ice
Plasma electrophoresis (albumin and globulin)	20 µL heparinized plasma (volume also included in the 320 µL CBC sample)	Spin whole blood at 2,000 g for 10 min at 10°C, remove supernatant (plasma)	Labeled plastic cryovials with internal O ring	Freeze on dry ice and store at -70°C	Send to Miami O/N on dry ice
Corticosterone	150 µL heparinized plasma	Spin whole blood at 2,000 g for 10 min at 10°C, remove supernatant (plasma)	Labeled plastic 1.5 mL cryovial (internal O-ring)	Freeze on dry ice and store at -20°C	Send to Cornell O/N on dry ice

Table 1. Analysis detail, sample containers, preservation techniques, and holding times for each type of sample/analysis/ endpoint to be conducted (priority samples highlighted in bold text). *Samples are priority level 2 and will be stored for potential analysis at a later date.

Analysis	Sample type (minimum volume)	Preparation details	Storage container	Storage conditions	Analysis lab/ sent details
Oxidative stress (GSH/GSSG; blood)	50 µL heparinized RBCs	Spin whole blood at 2,000 g for 10 min at 10°C, remove supernatant (plasma)	Labeled plastic 1.5 mL cryovial (internal O-ring)	Freeze on dry ice and store at -70°C	Analysis at CBL (Cayman kit)
Oxidative stress (GSH/GSSG; liver)	One 0.5 g piece of liver	Slice piece from whole organ, weigh it	Labeled plastic 2 mL cryovial (internal O-ring)	Flash freeze in liquid nitrogen and store at 70°C	Analysis at CBL (Cayman kit)
Oxidative damage (lipid peroxidation)	One 0.5 g piece of liver	Slice piece from whole organ, weigh it	Labeled plastic 2 mL cryovial (internal O-ring)	Flash freeze in liquid nitrogen and store at 70°C	Analysis at CBL (Oxford kit)
*CYP enzymes (liver)	One 0.5 g piece of liver	Slice piece from whole organ, weigh it	Labeled plastic 2 mL cryovial (internal O-ring)	Flash freeze in liquid nitrogen and store at 70°C	Analysis at CBL (exact method/kit TBD)
*PAH and metabolites	0.1 mL or more of bile	Collect bile with needle and syringe before removing liver; transfer to sample container	Solvent rinsed, baked, or trace clean Amber glass vial with PTFE lid	Freeze on dry ice and store at -70° C	Analysis by contract lab (TBD)
Histopathology	**GI tract, liver** (gallbladder emptied), kidney, spleen (half), **adrenal**, thyroid, heart, lungs, gonads, muscle tissue (one hind leg)	Collect bile, liver and spleen samples first (for other analyses), then fix in 10% NB formalin. Slice thick tissue if needed.	All organs in a single labeled jar (1 L) at least 10x volume NBF; decant and replace formalin after 24-hrs	Room temp	Analysis by contract lab (TBD)
*DNA damage (COMET assay); blood and liver cells	20 µL heparinized blood; 10 mg liver tissue	Remove 20 µL from vacutainer place in tube; place liver in tube on ice	Place in plastic tube.	Keep on ice, in the dark and assay in < 2 h	Analysis at CBL (needs immediate processing)

Table 1. Analysis detail, sample containers, preservation techniques, and holding times for each type of sample/analysis/ endpoint to be conducted (priority samples highlighted in bold text). *Samples are priority level 2 and will be stored for potential analysis at a later date.

Analysis	Sample type (minimum volume)	Preparation details	Storage container	Storage conditions	Analysis lab/ sent details
*Transcriptomics (blood)	500 µL of heparinized blood	Remove 500 µL from vacutainer and place in PAXgene tube; invert tube 8–10 times	PAXgene tube	Store upright at R.T. for 2–6 h, then store at 2–8°C for < 5 days. If a delay in shipping occurs, store at -20°C after 5 days.	Send to Hollings Marine Lab O/N on ice

CBC: complete blood count.
GI: gastrointestinal.
NBF: neutral buffered formalin.
PCV: Packed Cell Volume.
RBC: red blood cell
WBC: white blood cell.

Table 2. Specific endpoints to test in the blood chemistry panel and associated endpoints (University of Miami): Grouped samples in reptile panel noted by asterisks. Analytes are listed in order of priority.

Chemistry endpoint to test	Volume of plasma	Cumulative volume of plasma required
Plasma electrophoresis (albumin and globulins)	20 µL	One 320 µL plasma sample for both endpoints
Total protein (TP)	300 µL*	
Glutamate dehydrogenase (GLDH)	100 µL	A separate 100 µL plasma sample for GLDH
Gamma-glutamyl transpeptidase (GGT)	*	
Glucose (Glu)	*	
Uric acid	*	
Creatine phosphokinase (CPK)	*	
Aspartate aminotransferase (AST)	*	
Lactate dehydrogenase (LDH)	*	
Triglycerides	*	
Sodium (Na)	*	
Calcium (Ca)	*	
Chloride (Cl)	*	
Phos	*	
Cholesterol	*	
Alkaline phosphatase (ALkPhos)	*	

1.1.2 Turtle housing, maintenance, and monitoring during experiment

At least one day prior to dosing begins, turtles will be selected for the study from the pool of individuals and placed in individual 5.5 gallon glass tanks in one of two temperature-controlled water baths (98" long x 48" wide). A unique test identification (turtle ID number) will be placed on the outside of each tank so that individual turtles can be readily identified. Tanks will also be color coded to aid in quick identification of dose group (i.e., controls will have white tape, low dose green tape and high dose red tape). After e-tube insertion, turtles will be placed into the temperature-controlled water baths in individual 5.5 gallon tanks. Once placed in the external water bath, the heated external recirculating water will maintain their water temperature between 77–79°F. Individual thermometers will be placed in each water bath as well as in each individual 5.5 gallon tank to monitor water temperatures. Turtles from each of the three treatment groups will be placed in a random design between the two temperature-controlled water baths. UVA/UVB strip lights (on a 10:14 hour light:dark regime) and basking UVA/UVB heat ceramic bulbs will be placed above the water baths to maintain light quality and air temperature.

Each turtle will be weighed (body weight; g) and measured (carapace length; mm) before being placed in their tank. Each turtle will have a health monitoring data sheet assigned to it (see Attachment 1) to keep track of daily health (feeding / behavior / other visual observations) as well as an experimental monitoring data sheet (see Attachment 1) to record experimental

conditions such as daily feeding (amount, type and time), daily cleaning, and temperature and light regimes. The turtles will remain in their individual tanks for the duration of the experiment. As outlined in the housing and monitoring SOP #5, water will be changed and turtles will be fed and monitored for signs of stress on a daily basis. Turtle weight and length measurements will be taken daily before each dosing and at the end of the study before the turtle is euthanized and necropsied.

1.1.3 Oral dosing of oil and/or control solutions using the e-tubes

The toxicant in this study will be the Slick A oil to be provided by Abt Associates. Turtles will be randomly assigned to one of the three treatment groups using n = 15 turtles per group. Each turtle will be assigned individual test numbers that are placed on the outside of each tank in addition to a group (dose) color code. If sexes are known prior to the onset of dosing, treatment groups will be balanced for sex. The treatment groups for phase 4 will be as follows:

1. A control group
2. A 100 mg of oil per kilogram body weight group
3. A 1,000 mg of oil per kilogram body weight group.

Before the initiation of Phase 4, turtles will be allowed to recuperate from the e-tube surgery for a minimum of 48 hours prior to oil dosing. At the beginning of the test and daily for the 14-day period, all turtles will be briefly removed from their 5.5 gallon tanks (for approximately 2 min), weighed and re-dosed with the appropriate volume of oil based on the turtle's dosing group and weight. Body weight and carapace length measurements will be taken according to SOP #1 and recorded on the dosing data sheets (see Attachment 1). Any external sign of injury or issues with the e-tube will be noted on the data collection sheets.

To prepare the oil dose for the turtles the oil will be mixed with the food slurry and mixed to form a homogeneous mixture. This mixture is needed so that the oil does not retain onto the e-tube wall and that an accurate low dose can be provided. The food slurry is prepared with 30 mL of DI water and six small scoops of the powdered feed and well mixed. For the low dose 20 g of the food slurry is weighed into a solvent rinsed glass beaker after which 1 g of Slick A oil is also added. The mixture is well homogenized and the low dose animals weighed and their weight (in kg) multiplied by 2.1 provides the mg dose of the feed mixture to use (i.e., the target dose is 100 mg (0.1 g) oil and 2,000 mg (2 g) feed per kg turtle). For the high dose group 20 g of the food slurry is weighed into a solvent rinsed glass beaker after which 10 g of Slick A oil is also added. The mixture is well homogenized and the low dose animals weighed and their weight (in kg) multiplied by 3 provides the mg dose of the feed mixture to use (i.e., the target dose is 1,000 mg (1 g) oil and 2,000 mg (2 g) feed per kg turtle). To dose the turtles, the necessary amount of oil/food mixture will be drawn into a syringe. The weight of the oil/food mixture in the syringe before and after dosing will be monitored to calculate the exact dosing into the animal. This information will be recorded on the dosing data sheet and also in the laboratory book. To dose, the end cap from the e-tube will be removed and the end of the e-tube and cap wiped with ethanol. The oil/food mixture will be placed down the e-tube by inserting the luer lock syringe into the end of the e-tube. After the oil/feed mixture is added, a volume of 0.9% sterile saline equaling 3 mL per kg turtle body weight will also be inserted via syringe into the e-

tube to flush all of the oil/feed slurry into the stomach and also to prevent any future blockage of the e-tube. The use of the oil/feed mixture significantly minimizes the amount of residual oil left in the e-tube following dosing and flushes the slurry into the stomach and help ensure that the e-tube does not get blocked. Now full of sterile saline, the e-tube end-cap will be replaced and the turtle placed back into its individual glass aquarium. All times, weights/lengths of the turtles, and weights of the oil, feed and saline doses will be recorded on the daily dosing data sheets.

Dosing will continue for a maximum of 14 days, but may be less if turtle health deteriorates to the level that they cannot be treated by a veterinarian and retained in the study or if the turtle removes its e-tube or the e-tube becomes unusable. If the turtle removes the e-tube and there is no sign of stress we will attempt to keep the turtle in the study by using the removed e-tube to orally via gavage dose the turtle. Condition of each turtle will be monitored at least once daily and recorded in the daily health and monitoring data sheets (see Attachment 1). We will note any considerable changes in weight and overt signs of stress such as lethargy, persistent recumbency, or decreased intake of food or fecal production.

The health and monitoring datasheet will record the times each turtle is fed and their water is changed so that accurate assessments of feeding and feces production can be noted. Estimated feces production, the amount of feeding, and the approximate volume of regurgitated/excreted oil will be recorded daily. The appearance of an oil sheen in the water will be noted and attempts will be made to score the amount of oil using a visual score. In addition, a daily health assessment of each turtle will be recorded on this data sheet noting visual external appearance (skin color etc.), observations as to infection or injury at the e-tube insertion site and assessments of behavior. The dates and times of oil dosing and body weight / length on days of dosing will be noted on the dosing datasheet (see Attachment 1).

1.1.4 Euthanasia, blood sample collection and necropsy

It is anticipated that necropsies will be performed at termination of the dosing, however, if turtle condition deteriorates such that an individual cannot be retained in the study, it will be necropsied prior to termination of the experiment. In addition, if during the course of the study animals remove their e-tubes these animals will immediately be removed from the study and necropsied at the time of noted removal of the e-tube. Signs of damage by the e-tube and or infection at the site of insertion will be visually inspected for in all turtles during necropsy.

Each turtle on trial at the completion of the dosing will be weighed and the carapace length recorded on the necropsy data sheet. They will be photographed ventrally and dorsally (note photographs in the Photographic log datasheet) and then euthanized according to SOP #7: *Euthanasia by Cervical Dislocation (decapitation followed by pithing of the brain)*. Blood will be collected via cardiac puncture according to SOP #3b prior to euthanasia and processed according to the endpoints listed in Table 1. In addition, a necropsy will be conducted. The necropsy of each turtle will follow the procedures in SOP #8. A ruler to show scale and a card with the turtle's identity indicated on it should be included in each photograph taken before and during necropsies.

During necropsy, the heart, kidneys, liver, lungs, GI tract, spleen, thyroid, thymus, gonads, and adrenal glands will be collected. All organs will be assessed for gross abnormalities. If abnormalities are present, a photograph of the abnormality will be taken. A ruler to show scale and a card with the turtle's identity indicated on it should be included in the photograph. If the abnormality is a discrete lesion, it will be removed with a section of undamaged tissue attached and placed in 10% NBF. All abnormalities and/or lesions will be recorded on the necropsy sheet. Once assessed, all organs will be placed in an appropriately labeled specimen jar containing 10% NBF for subsequent histopathological analyses. Note, before the liver is placed in specimen jar, sub-samples should be removed, as discussed below, for subsequent analyses. Also, the GI tract should be flushed of any consumed food and cleaned if diameter is greater than 0.5 cm before adding to specimen jar (see discussion below). If edema is seen in the GI tract, wall thickness should be assessed. No sections placed in the NBF should be more than 5 mm thick. If tissues or lesions are larger than this the tissues will be sliced (bread loaf) to allow formalin perfusion and adequate fixation.

If present, bile may be collected for chemical analysis of PAH metabolites. Before the removal of the liver the gall bladder will be located and all of the contents removed into a cryovial and flash frozen in liquid nitrogen and stored at -80°C. At least 0.1 mL of bile is needed, but more should be collected if possible. If volumes are less than 0.1 mL, individual samples may be pooled for PAH metabolite analyses at a later date.

After removal of bile, the whole liver is removed and weighed. After weighing the whole liver, four liver tissue samples (approximately 2 g each) will be immediately sub-sampled from the right (non-gall bladder containing) lobe, placed in cryovials and flash frozen in liquid nitrogen (or dry ice) and stored at -80°C for potential future assessment of cytochrome P450 (CYP450) enzyme activity, oxidative stress endpoints (i.e., GSH and lipid peroxidation), and PAH analyses. After collection of these samples, all remaining liver tissue will be placed in the specimen jar containing 10% NBF. If the thickness of the liver is too great, before placing in 10% NBF the liver will be scored using a scalpel to allow adequate perfusion of the 10% NBF into the tissue.

For the GI tract, the whole organ will be removed intact and at regular intervals (at least 5 times) over the length of the tissue will be scored with the scalpel blade to open up the tissue to allow removal of any consumed food that may be in the upper and lower tract and also to ensure adequate fixative penetration. The tissue will be slit vertically, rinsed thoroughly in phosphate buffered saline, and assessed for potential edema and lesions. Once assessments are complete the GI tract will be placed in the histology specimen jar containing the rest of the organs in 10% NBF.

After all samples are collected any remaining pieces of tissue will be placed into the body cavity of each respective turtle. The carcass will be placed into a labeled plastic bag for archiving and placed in the -20°C freezer.

Necropsy Datasheets (Attachment 1) will be used to document necropsy observations and tissue samples (excluding blood) for each turtle. The date and time of necropsy, the weight of each organ/tissue sample removed, a description of any gross abnormalities (in addition to digital image file information), and the disposition of tissue slices (buffered formalin, cryovial in dry

ice/liquid nitrogen) will be recorded. Necropsy Datasheets provide blank spaces for recording additional samples and observations not otherwise documented elsewhere.

1.1.5 Blood collection and processing

As described above, at the end of the 14-day period turtles will be weighed and carapace length recorded according to SOP #1. Six Five of the 15 turtles in each dose group will have blood samples taken at the conclusion of the study only. As noted above, a sub-set of n = 10 turtles will undergo ACTH challenge to examine adrenal responses to oil dosing. Prior to ACTH dosing, blood will be collected according to SOP #3b for analysis of all routine blood chemistry and biological endpoints and to establish a baseline corticosterone level for the ACTH challenge. Turtles undergoing ACTH challenge will receive a 50 IU/kg dose after the initial blood draw, and a final blood sample will be taken after 60 min to assess final corticosterone levels.

All animals, including those that undergo ACTH challenge, will then be euthanized according to SOP# 7 and necropsied according to SOP #8. Blood will be collected directly from the heart immediately prior to euthanasia via cardiac puncture so that a pure blood sample can be obtained. Blood samples for turtles undergoing ACTH challenge will be taken prior to ACTH dosing. Blood samples will be collected according to SOP #3b and recorded on the Blood Sampling datasheet (Attachment 1). Blood samples will then be processed according to SOP #4 and the resultant plasma samples stored at -80°C. Plasma preparations will be divided into at least three aliquots for sending for different analyses (i.e., Aliquot 1 is up to 150µl and will be sent to Cornell University for corticosterone analyses on dry ice O/N; Aliquot 2 is 100 µL and Aliquot 3 is 320 µL which will both be sent on dry ice O/N to the University of Miami for plasma proteins and blood chemistry panels respectively, any remaining plasma will be placed in Aliquot 4 and archived at CBL at -80°C. A portion of whole heparinized blood (before plasma preparation) will be used to prepare the blood smears at CBL (3 slides for each individual). For blood smears, three slides will be prepared per turtle by CBL using well-mixed, heparinized whole blood according to the SOP #2, and an additional three slides will be prepared at the University of Miami with two of these slides being stained by New Methylene Blue (NMB); therefore, 100 µL whole blood will be collected and sent to the University of Miami via O/N FedEx for their PCV and blood smears. In addition, of the three slides prepared by CBL, one slide will be stained with Diff-Quick by University of Miami. After completion of slide evaluation at University of Miami, all 6 slides will be shipped to Nicole Stacy, where she will stain the remaining three unstained slides with Wright-Giemsa. This gives a total of 6 whole blood smears per turtle. Whole blood smears will be used for WBC estimates, WBC and RBC morphologic assessments. Smears will require less than 10 µL of heparinized whole blood. An additional volume (< 50 µL) of whole heparinized blood will be used for the PCV assessments at CBL according to SOP #9. Also, a further 100 µL aliquot of whole heparinized blood will be collected and sent to University of Miami for the hemoglobin assay. For the GSH/GSSG analyses in blood to be completed at CBL the blood tube used to prepare the plasma (containing the pellet) will be frozen (i.e., this is at least 400 µL of heparinized RBCs).

If enough whole heparinized blood remains, exactly 0.4 mL will be transferred to the Qiagen tube containing the blood stabilization solution. Immediately after blood transfer, gently invert

the PAXgene tube 8–10 times, then stored upright at room temperature for 2–6 hours before moving to a refrigerator (2–8°C).

For a list of all blood/plasma samples to be collected and their respective analyses see Table 1. Blood sample collection information will be documented using the datasheet provided in Attachment 1: *Blood Sample Inventory Datasheet*. This datasheet will be turtle-specific and used to document all blood samples collected from each turtle for each collection time point.

1.1.6 Documents and records

Toxicity testing information documented in notebooks, data sheets, photograph files and logs, Chain of Custody forms, and shipping forms will follow procedures described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP). Additional data sheets specific to this study are provided below. Please refer to the QAPP for all instructions pertaining to recordkeeping, sample label protocols, and document retention requirements.

1.1.7 Table summarizing SOPs

SOP #	Procedure	SOP #	Procedure
1	Turtle weight and carapace length	8	Necropsy for blood and tissue collection
2	Preparation of whole blood smears	9	Packed Cell Volume
3a	Subcarapacial venous sinus blood collection	10	Protein (BCA) assay
3b	Cardiac puncture blood collection	11	Tissue homogenization procedure
3c	Dorsal coccygeal vein blood collection	12	Glutathione quantitation (tGSH and GSSG) in blood cells
4	Preparation of blood plasma samples	13	Lipid peroxidation assay (TBARS) for plasma
5	Housing, feeding and monitoring of turtles	14	Lipid peroxidation assay for liver
6	Surgical procedures for the placement / insertion and monitoring of the e-tubes and drilling for cardiac puncture access during necropsy	15	Total antioxidant assay (TA) for liver (SOP#15a) and plasma
7	Euthanasia by cervical dislocation (decapitation followed by pithing of the brain)	16	COMET assay for blood cells (SOP#16a) and liver tissue

Standard Operating Procedure: Turtle weight and carapace length
SOP#1

1. Scope and Applicability

This document outlines the procedures by which turtle weight and carapace length is assessed before and after daily dosing.

2. Health and Safety

Non-slip footwear and PPE should be worn when handling turtles.

3. Personnel Qualifications

All persons involved in turtle handling will have experience restraining turtles and will have completed all necessary animal care training as prescribed by their institutional IACUC.

4. Materials Required

1. Water proof markers
2. Dosing or necropsy datasheet
3. Balance (precise to at least 0.1 g)
4. Container or turtle holding bag

5. Turtle Weight and Length measurements

1. Confirm that top-loading balance is in proper working order and is properly calibrated.
2. Capture and restrain turtle appropriately.
3. Carefully carry turtle to weighing station.
4. Record turtle ID on turtle dosing or necropsy data sheet.
5. Using calipers measure the carapace length and record on the turtle dosing or necropsy data sheet.
6. Place empty container (large weigh boat) on the calibrated scale and tare balance for the containers weight.
7. Carefully place the turtle inside the container and ensure the container is secured.
8. Record weight on data sheet.
9. Remove turtle from container/balance.
10. Place the turtle back into the appropriate housing or continue to dosing or necropsy.
11. Repeat until all of the turtles have been weighed

Standard Operating Procedure: Preparation of Whole Blood Smears
SOP#2

1. Scope and Applicability

The purpose of this document is to describe the procedure for preparing thin blood smears for complete blood counts (CBC), for red blood cells (RBC), and for white blood cell (WBC) analyses.

2. Health and Safety

Workers should be aware that they are using glass slides and take usual precautions to avoid cuts and ensure all broken slides are disposed of correctly. Although the blood volumes used are generally < 20 µL workers should also be aware of all cleanup procedures for turtle blood.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all training or be able to demonstrate appropriate experience working in a laboratory setting and have completed the institutional IACUC requirements for working with turtles.

4. Materials and Equipment

1. Cleaned beveled edge glass slides with frosted ends
2. Pre-printed labels according to appropriate laboratory work plans
3. Micropipette (5-20 µL)
4. Pipette tips
5. Sample preparation data sheet
6. Blow dryer

5. Procedure

1. Glass slides should be clean, grease and scratch free and have smooth edges without any cuts.
2. Place label on frosted end nearest edge; cross-check label with sample to be applied.
3. Using a micropipette, place a small drop of whole heparinized blood (2-5 µL) close to the end of the slide, approximately 0.5-1.0 cm from the label.
4. Then place the short edge of another slide (the smearing slide), flat against the bottom of the slide approximately 0.5 cm further along the slide (away from the label) than the blood spot.
5. Hold the 'smearing slide' at an angle of 45° from the surface of the flat slide.
6. Move the edge of the 'smear slide' toward the blood spot until it touches.

7. Allow the surface tension of the blood spot to move the blood along the width of the 'smearing' slide.
8. Smoothly draw the blood along the length of the slide to create a feathered edge that almost reaches the other end of the slide.
9. Check that the edge is feathered and that there are no streaks. If streaks are present repeat the procedure with a fresh-labeled slide.
10. Six blood smear slides must be produced for every blood sample (3 prepared at CBL and 3 prepared at University of Miami).
11. Allow the smears to air dry or if necessary the slides can be dried under a low air stream from a blow dryer.
12. Once slides are dry they can be stored in a slide box at room temperature.
13. If slides are broken or unusable they should be disposed of appropriately.

Standard Operating Procedure: Subcarapacial venous sinus blood collection (#3a), cardiac puncture blood collection (#3b) and dorsal coccygeal vein blood collection (#3c). SOP#3a/b/c

1. Scope and Applicability

The purpose of this document is to describe the procedure for preparing needles and syringes and drawing blood from turtles from the subcarapacial venous sinus vein which may be used for the corticosterone analyses (i.e., all of Phase 2, and maybe for the corticosterone analyses in Phase 3 and the ACTH challenge/corticosterone analyses in Phase 4) or alternatively blood may be drawn using the dorsal coccygeal vein (SOP #3c). NOTE, whichever method is used needs to be consistent across all individuals for the particular experiment (i.e., for all of Phase 2). For all other blood analyses in Phase 3 and 4, blood draws will follow SOP#3b using the cardiac puncture method. The anti-coagulant that will be used will be lithium heparin.

2. Health and Safety

Workers should be familiar with procedures for first aid for needle sticks, as well as sharps and biohazardous waste disposal.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all training or be able to demonstrate appropriate experience working in a laboratory setting. All personnel handling turtles should be trained according to the Institutional IACUC guidelines.

4. Materials and Equipment

1. Syringes (1.0-3.0 mL)
2. 22, 23 and 25 G needles
3. 100 IU lithium heparin solution in 0.9% NaCl (ensure that all heparin is expelled from the needle prior to use)
4. 30% ethanol
5. Hydrogen peroxide
6. Sterile cotton swab or gauze
7. Cryopen or permanent marker
8. Labeled microtainers (400-600 µL, or 1.3 mL) or vacutainers (2.0-3.0 mL) as appropriate for the analysis to be completed (i.e., heparinized treated samples)
9. Labeled microcentrifuge tubes (0.5 and/or 1.5 mL) or cryovials (1-2 mL)
10. Micropipettes (100-1000 µL)
11. Pipette tips
12. Blood Sample Inventory Datasheet
13. Refrigerated centrifuge capable of speeds up to 5000 g
14. Ice bucket and ice

5. Procedure

Syringe preparation

1. Aseptically open syringe and attach a fresh needle if there is not a pre-attached needle. Break the plunger seal by drawing and depressing the plunger prior to use.
2. Needles and syringes can be prepared prior to sampling several turtles.
3. Draw a small volume (sufficient to fill the needle only) of lithium heparin into the syringe.
4. Move the plunger down the length of the needle, and syringe, then expel excess anticoagulant.
5. Ensure there is no fluid in the syringe and no droplet on the end of the needle.
6. Store the needle and syringe at room temperature until use.

SOP#3a: Subcarapacial venous sinus blood collection method

1. Check Turtle ID against the label on the microtainer, vacutainer and microcentrifuge or cryovial tubes into which the sample will be decanted.
2. Place the turtle on its plastron on a flat surface on the edge of the bench.
3. Secure the body of the turtle with personnel No. 1 who should also record all data and ensure samples are placed into the correct containers, personnel No. 2 will collect the blood.
4. The turtle head should be secured and an ethanol swab used to sterilize the area on top of the head and towards the neck/body of the turtle where the subcarapacial venous sinus is located.
5. Locate the position for the subcarapacial venous sinus blood draw and adjust the turtle's position accordingly to ensure that the vein is accessible.
6. Insert the needle (25 G needle) into body of the turtle, upwards toward the carapace at an approximate 20-50° angle.
7. If the needle is properly inserted into the vein there will be a small flush of blood into the syringe. Take note if it is pure blood, lymph contaminated blood or lymph.
8. If the draw is pure blood then gently pull back on the syringe to fill.
9. Turn the syringe gently if flow begins to slow.
10. If lymph or lymph contaminated blood is observed stop collection, note the sample attempt and repeat until a pure blood sample is obtained. If a pure blood sample cannot be obtained after several attempts, proceed with collection of a sample, and make a note of the lymph contamination in the collection sheet.
11. If more than one syringe volume is required or samples need to be repeated as outlined in step 10 then gently remove the syringe from the needle and replace.
12. Repeat steps 9 and 10.
13. When sufficient volume is obtained place a cotton swab over the injection site and gently apply pressure as the needle is removed.
14. After approximately 90 seconds check to ensure bleeding has clotted.
15. Remove the needle from the syringe and carefully decant blood into a labeled microtainer, or vacutainer as appropriate.

16. Record blood volumes obtained on the blood sampling datasheet and make a note if lymph contamination occurred and an estimate of lymph:blood volumes.
17. Gently invert the vacutainer tube 10 times prior to separating blood into aliquots for different sample preparations. Place samples at room temperature or in a container on ice (not directly on the ice) or in the fridge at 4°C as appropriate (specified in test-specific work plan).
18. Centrifuge within 1 hour if centrifugation is required for the sample (i.e., preparation of plasma). Make note of the color of the plasma to determine if hemolysis occurred (i.e., yellow plasma or pink/red tinted plasma).
19. Store samples as appropriate (e.g., 4°C, freeze samples on dry ice and store at -80°C; specified in test-specific work plan).
20. Ship samples to appropriate laboratory as specified in test-specific work plan.

SOP#3b: Cardiac puncture method

1. Check Turtle ID against the label on the microtainer, vacutainer and microcentrifuge or cryovial tubes into which the sample will be decanted.
2. Place the turtle on its plastron on a flat surface on the edge of the bench.
3. Secure the body of the turtle with personnel No. 1 who also should record all data and ensure samples are placed into the correct containers, personnel No. 2 will collect the blood.
4. Immediately before euthanasia the turtle will be prepped for the cardiac blood draw. For the snapping turtles the needle will be placed between scutes. For the sliders a hole will be drilled in the plastron to allow the needle to enter the body cavity.
5. Insert the needle (23 G needle) into body of the turtle toward the heart at an approximate 90° angle.
6. Gently pull back on the syringe to fill.
7. If more than one syringe volume is required or samples need to be repeated as outlined in step 27 then gently remove the syringe from the needle and replace.
8. Repeat steps 27 and 28.
9. Remove the needle and carefully decant blood into a labeled microtainer, or vacutainer as appropriate (specified in test-specific work plan).
10. Record blood volumes obtained on the blood sample inventory datasheet.
11. Gently invert the vacutainer tube 10 times prior to aliquoting for different sample preparations. Place samples at room temperature or in a container on ice (not directly on the ice) or in the fridge at 4°C as appropriate. Centrifuge within 1-2 hours if centrifugation is required for the sample (i.e., preparation of plasma). Make note of the color of the plasma to determine if hemolysis occurred (i.e., yellow plasma or pink/red tinted plasma).
12. Store samples as appropriate (e.g., 4°C, freeze on dry ice and store at -80°C; specified in test-specific work plan).
13. Ship samples to appropriate laboratory as specified in test-specific work plan.

SOP#3c: Coccygeal vein method

1. Check Turtle ID against the label on the microtainer, vacutainer and microcentrifuge or cryovial tubes into which the sample will be decanted.
2. Restrain the turtle on a table, plastron down, with its head covered with a towel to minimize stress to the animal.
3. Secure the body of the turtle with personnel No. 1 who should also record all data and ensure samples are placed into the correct containers, personnel No. 2 will collect the blood.
4. The person restraining the turtle will extend the tail away from the body and down to expose the cranial end of the coccygeal vein and an ethanol swab used to sterilize the area
5. Personnel No. 2 with turtle venipuncture experience, will use a 22, 23 or 25G, 1 inch needle, with either a 1 mL or 3 mL syringe attached (depending upon the blood volume required) to obtain the blood sample.
6. The needle is inserted at an approximate 90° angle in the dorsal midline, aiming for the coccygeal vertebrae. Once vertebrae is touched, apply negative pressure and pull needle back slowly until the dorsal coccygeal vein is reached.
7. Gently pull back on the syringe to fill.
8. Remove the needle and carefully decant blood into a labeled microtainer, or vacutainer as appropriate (specified in test-specific work plan).
9. Record blood volumes obtained on the blood sample inventory datasheet.
10. Gently invert the vacutainer tube 10 times prior to aliquoting for different sample preparations (if appropriate). Place samples at room temperature or in a container on ice (not directly on the ice) or in the fridge at 4°C as appropriate. Centrifuge within 1-2 hours if centrifugation is required for the sample (i.e., preparation of plasma). Make note of the color of the plasma to determine if hemolysis occurred (i.e., yellow plasma or pink/red tinted plasma).
11. Store samples as appropriate (e.g., 4°C, freeze on dry ice and store at -80°C; specified in test-specific work plan).
12. Ship samples to appropriate laboratory as specified in test-specific work plan.

Standard Operating Procedure: Preparation of blood plasma samples
SOP#4

1. Scope and Applicability

The purpose of this document is to describe the procedure for separating blood for assay and storage following collection of blood according to *SOP#3, Subcarapacial venus sinus blood collection (#3a), cardiac puncture blood collection (#3b) or coccygeal vein blood collection (#3c)*. Consult the appropriate work plan in advance and ensure that all tubes for RBCs or plasma are pre-labeled.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including (if handling the turtles) the Institutional IACUC.

4. Materials and Equipment

1. Blood filled microtainers or vacutainers.
2. Blood sample inventory datasheet
3. Pre-labeled micro-centrifuge tubes or cryovials
4. Ice, dry ice as appropriate
5. Micropipettes (100-1000 µL)
6. Pipette tips
7. Transfer pipettes
8. Centrifuge capable of spinning at (1000-5000 g)

5. Procedure

1. Place labeled blood filled microtainer or vacutainer tubes into an appropriate refrigerated (4°C) centrifuge.
   a. Spin vacutainer tubes at > 2,000 g for 10 minutes.
   b. Spin speed and time may be modified according to manufacturer's specifications; If different then above, record on datasheets or laboratory notebook
2. Check that blood is sufficiently separated.

3. Note if there is any lysis (i.e., color of the supernatant e.g., yellow/pink/red) and record on the blood collection data sheet.
4. Using the micropipette, aliquot volumes of heparinized plasma or serum into the appropriate cryovials or microcentrifuge tubes.
5. Record the samples prepared on the blood sample inventory form.
6. Store as directed in the test-specific work plan.
7. Repeat until all samples have been processed.

Standard Operating Procedure: Housing, feeding, and monitoring of turtles
SOP#5

1. Scope and Applicability

The purpose of this document is to describe the procedure for housing the turtles, caring and feeding them, and monitoring their overall health during a study. The snapping turtles and all experimental (phase 2-4) red-eared sliders will be housed in the temperature-controlled rooms in individual 5 or 10 gallon glass aquaria.

2. Health and Safety

Workers should be familiar with working with turtles, including risk of bacterial infections from handling turtles and risk of turtles biting during handling.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all training or be able to demonstrate appropriate experience working in a laboratory setting. All personnel handling turtles should be trained according to the Institutional IACUC guidelines.

4. Materials and Equipment

1. 5.5 gallon glass tanks
2. 2 large fiberglass water baths (48" (width) x 98" (length) x 12" (depth)
3. Two 250 Gallon fiber glass header tanks
4. Small (150W) ceramic heaters (6 per water bath)
5. Shop lights; 2-strip 48" light fixtures (4 per water bath)
6. Reptisun UVA/UVB 48" lamps
7. Extension cords (4 x 10 outlet shop lights)
8. 20 300-500W titanium heaters
9. 80 2" bricks and 16 4" blocks
10. 8 x 2 x 6 4" wood strips
11. 35 8 ft 2x4s for wooden frames to hold light fixtures/heaters
12. Timers for lights (10 hours on; 14 hours off)
13. Temperature probes for each water bath, header tank, and individual turtle tank
14. 1.5" and 1" PVC piping and fittings/heaters
15. Cord/string to hang light fixtures/heaters
16. Colored tape and labels for tank/turtle ID#

17. 3 x pool pump and tubing/hose for vacuum removal of water
18. Pumps/tubing for adding water to tanks
19. Storage tanks/sand/activated charcoal system for oil/water filtration
20. Live mealworms
21. Complete reptile pellet feed
22. Gloves (nitrile or latex)
23. Daily health and environmental monitoring datasheets
24. Temperature controlled room
25. Neosporin
26. Cotton swabs

5. Procedure

Feeding of turtles

1. Turtles are fed daily (at least 3-4% weight of body mass) live large mealworms (Wednesdays and Fridays) or, if mealworms not available, commercial growth pellet feed (Mondays/Tuesdays/Thursdays/Saturdays and Sundays).
2. The following morning before daily tank cleaning, tanks are inspected for remaining food. If food is remaining then it is noted on the daily health monitoring datasheet and attention will be placed on the individual to assess for length of non-feeding and any other signs of stress.

Cleaning of turtles

1. Turtles are cleaned daily.
2. For all individual experimental tanks, water is removed daily using a pump, suction and tubing. New water (which has been heated to the appropriate temperature of 26°C) is pumped from the reservoir tank into each glass aquaria until approximately 1/3 full.
3. Oil dosed turtle water will be filtered through an oil removal system (sand/activated charcoal or if too concentrated will be sent straight for hazardous waste disposal) before draining to the sewage system.

Environmental monitoring and turtle health monitoring

1. Temperature-controlled water baths will be checked daily to ensure that temperatures are maintained.
2. All glass aquaria will also be individually monitored for temperature and recorded on the environmental monitoring datasheets.
3. At least once daily each turtle is inspected for any signs of stress or poor health. If a wound is observed, it will be daily treated with Neosporin applied using a cotton swab. The exposed skin of the turtles will be looked at for any signs of bacterial, parasitic infections or redness/swelling and edema. If any signs of stress occur, this will be noted in daily health monitoring datasheets.
4. During experimental procedures, turtle weight and carapace length will be measured daily.
5. Any change in the turtles' normal behavior will be noted.
6. Before water changes, the presence of uneaten food and /or feces will be noted.

Standard Operating Procedure: Surgical procedures for the placement / insertion and monitoring of the e-tubes and drilling for cardiac puncture access during necropsy SOP#6

1. Scope and Applicability

The purpose of this document is to describe the surgical procedures for placement/insertion of e-tubes and the drilling of the cardiac port for access to heart for cardiac puncture.

2. Health and Safety

Workers should be familiar with procedures for turtle handling, biohazardous waste disposal and cleanup.

3. Personnel Qualifications

The e-tube procedure will be carried out by trained veterinarians.

4. Materials and Equipment

1. E-tube datasheets
2. E-tube
3. Balance
4. Propofol
5. Meloxicam
6. Lidocaine
7. Scalpel
8. Hemostat
9. Stich kit

5. Procedure

1. E-tube procedure should be complete at least 48 hours prior to start of any experiment, to allow for appropriate recovery.
2. Using proper handling techniques, remove the turtle from the container.
3. Before e-tube insertion, weighed turtle and note the heart rate.

4. To start the procedure, inject turtles with either 2.5 mg/kg propofol (red-eared sliders) or 5 mg/kg propofol (snapping turtles) and note the time of administration.
5. When turtles are determined to be under sedation (again note the heart rate), the surgery can be initiated with an injection of meloxicam (0.2 mg/kg I.M.).
6. For the placement of the e-tubes the distance from the insertion site to mid body is measured and this measurement used to determine how much of the tube will be put into the esophagus/stomach. The incision location will be caudal cervical region on left side just cranial to carapace margin.
7. Sterile technique and sterile preparation of surgery site will be performed.
8. A hemostat will be inserted through the mouth and into the esophagus to the incision location and pressure used to push the tissue laterally causing the hemostat tip to be easily palpable. A scalpel blade will be used to incise the skin and esophagus, exposing hemostat tips. The end of the esophageal tube will be grasped in hemostats and retracted to mouth, then the tube tip will be redirected toward the stomach. The hemostats will then be used to guide the tube tip down the esophagus and towards the stomach.
9. The tube will be secured to the skin with purse string and Chinese finger locking suture pattern with non-absorbable, monofilament sutures. The tube will be secured to the shell with non-heating epoxy or similar material to affix to dorsal aspect of carapace.
10. The tube will be filled with sterile saline and the external end of tube will be secured shut with a removable plug.
11. After surgery has been completed a sharpie pen mark is placed on the e-tube next to the sutures so that it can be assessed if the tubes move (i.e., the turtles pull them out).
12. Lidocaine (2 mg/kg) is I.M. injected at the external e-tube site, the time is noted, the heart rate monitored and the turtle placed in recovery and observed until it has regained all normal behavior, at which time it will be placed into its individual tank containing a minimal amount of water (so that it does not cover the e-tube site).
13. Monitor turtle closely (at least twice a day) for any signs of stress or abnormal behavior for the next 48 hours. Also inspect the e-tube site.

Standard Operating Procedure: Euthanasia by Cervical Dislocation (decapitation followed by pithing of the brain)
SOP#7

1. Scope and Applicability

The purpose of this document is to describe the procedure for euthanasia of turtles at the completion of the experimental period. Euthanasia will be performed according to guidelines published by the American Veterinary Medical Association and the approved IACUC protocol. Generally a minimum of two people are required for this procedure to ensure minimal stress to the animal. Cervical dislocation is required for these experiments as some endpoint measurements could be adversely affected by injectables or inhalation anesthetics.

2. Health and Safety

Workers should be familiar with procedures for turtle handling, biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in this procedure will have be experienced in cervical dislocation and/or trained in euthanasia and immobilization and have completed the Institutional IACUC.

4. Materials and Equipment

1. Blood sample inventory and necropsy datasheets
2. No. 20 scalpel
3. Long handled needle

5. Procedure

1. Using proper handling techniques, remove the turtle from the container.
2. Stabilize turtle to reduce movement (Personnel 1).
3. Carefully stretch out the neck and locate vertebrae (Personnel 1).
4. Personnel 2 will immediately in one movement use the scalpel to completely separate the head from the neck and immediately after will take the head and insert the needle to pith the brain. Death can be assessed immediately.

Standard Operating Procedure: Necropsy for blood and tissue collection
SOP#8

1. Scope and Applicability

The purpose of this document is to describe the procedure for necropsy for blood and tissue collections for analyses. Necropsy will immediately follow euthanasia (SOP#7) and blood collection (SOP#3b). If required, blood samples will be processed according to SOP#4. Blood and tissue analyses will be conducted according to the appropriate work plan. Consult the work plan to determine storage conditions, appropriate volumes of blood and masses of tissue needed to meet the requirements for testing.

2. Health and Safety

Workers should be familiar with procedures for turtle handling, biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in necropsies should have completed all required training or be able to demonstrate appropriate experience conducting necropsies and working with biohazardous materials. All personnel handling turtles will be trained by the Institutional IACUC.

4. Materials and Equipment

1. 1-3 mL syringes
2. 25 G needles (for subcarapacial venus sinus collection prior to necropsy where relevant i.e., ACTH study baseline blood draw)
3. 23 G needles for cardiac puncture (one per turtle)
4. Lithium heparin solution
5. Labeled microscope slides
6. Phosphate buffered saline (PBS) or 0.9% sterile saline
7. 2.0-5.0 mL plastic vials with caps such as cryovials
8. Glass vials for bile samples
9. Vortex
10. 500 µL to 1.7 mL snap-lock plastic microcentrifuge tubes
11. 1-10, 2-20, 20-200 and 100-1000 µL pipette tips
12. 1-10, 2-20, 20-200 and 100-1000 µL micropipettes
13. 400 µL to 1.0 mL lithium heparin microtainers

14. 400 µL to 1.3 mL lithium heparin microtainers
15. 2.0-4.0 mL lithium heparin vacutainers
16. Medical dissection scissors (large and small straight blade)
17. Scalpel blades and handles
18. Forceps
19. Hemostats
20. Cotton swabs
21. Small dissection scissors (small straight and curved blades)
22. Laboratory timer
23. Digital camera and appropriately labeled SD card and stand
24. Dremel tool and blades
25. Large and small cutters
26. Three-decimal place balance (Sartorius CP 432S or similar)
27. 10% neutral buffered formalin (NBF)
28. Cryo boxes (5 x 5 x 3 inches or similar) with dividers
29. Liquid nitrogen
30. Kimwipes
31. Labels for vials (preferably cryobaby or similar to withstand liquid nitrogen)
32. Access to a -80° C freezer
33. Dry ice
34. Wet ice
35. Liquid nitrogen
36. Printed sample inventory sheet and matching sample ID labels
37. Necropsy datasheets
38. Blood sample inventory datasheets
39. Photolog datasheet
40. Ziploc or biohazard bags for carcasses
41. Containers for formalin samples
42. Cryopens and/or permanent markers

5. Procedure

Before beginning ensure all sample containers, vials, tubes, and slides are labeled and all labels have been cross-checked with the sample inventory. Ensure sufficient photographic records are kept to show all abnormalities in situ or once the organs are removed, as appropriate. Make sure the turtle ID number and date are clearly visible in each photograph.

Blood collection prior to anesthesia

1. Weigh turtle and record carapace length (as detailed in SOP #1)
2. Euthanize turtle by cervical dislocation following SOP#7
3. Collect blood according to SOP#3b
4. When sufficient blood volume is collected to meet all of the study requirements, or no further blood can be obtained the carcass is ready for dissection.
5. Record the number of blood tubes collected.
6. Process blood as per SOP#4.
7. Record sample ID information on the appropriate data sheet.
8. Store and aliquot blood according to the appropriate work plan.
9. Before necropsy take digital pictures of the turtle dorsally and ventrally on the score sheet that includes the label of the turtles ID # and a ruler for scale. Record the photograph on the photographic log datasheet.

Necropsy

1. Using the dremel tool / blade cut the carapace to separate the two to allow access to the body cavity.
2. Open the body cavity being careful not to puncture or move organs.
3. Note any abnormalities on the necropsy data sheet.
4. Collect blood according to SOP#3b. Try to collect as much blood as possible.
5. Following blood draw note any abnormalities on the necropsy datasheet
6. Take digital image of the all organs *in situ* upon initial opening after cardiac blood draw. Ensure that the study ID, turtle ID and date are clearly visible in the photograph. Record the photograph on the photographic log datasheet.
7. Locate the gall bladder and insert a 25G needle to remove bile.
8. Place bile in labeled glass vial, record volume and immediately place in liquid nitrogen to freeze.
9. After bile is frozen place in -80°C for long term storage.
10. Remove the liver and weigh the whole organ and record on the necropsy datasheet.
11. Take portions of the liver (3 x 0.5 g) for biochemical/molecular analyses by placing in labeled cryovials and immediately place in liquid nitrogen, record in datasheet.
12. After freezing liver portions in liquid nitrogen place frozen tissues at -80°C for long term storage.
13. Place the rest of the liver, after scoring to ensure proper fixing, in 10% NBF for histological analyses. No sections taken should be more than 5 mm thick. If lesions are larger than this, slice the tissues (bread loaf) to allow formalin perfusion and adequate fixation

14. Cut out the lungs and heart and separate heart from lungs.
15. Assess lungs for any abnormalities, weigh, and place with the liver in specimen jar with 10% NBF
16. Before weighing heart, open heart chambers, wash with sterile saline to remove blood and clots, and pat dry (repeat if necessary)
17. Once weighed, assess heart for any abnormalities and place in the 10% NBF specimen jar.
18. Next remove thyroid, located in the neck, just anterior to blood vessels near the clavicle and place in 10% NBF.
19. Cut and remove the whole GI tract including spleen (from throat through anus)
20. GI tract should be sliced vertically, and rinsed with sterile saline to remove all consumed food.
21. Check the GI tract for lesions (particularly at the e-tube insertion site), parasites, and edema. Then, prepare sections for histopathology:
    i. Parasites: If identification is required then some can be removed and fixed, otherwise the rest should be left in place, particularly if they are attached.
    ii. Lesions: Cut all lesions into pieces no more than 0.5 cm thick. Length is not important. Include an edge with some normal tissue.
    iii. Edema: If edema is observed, measure wall thickness using calipers.
22. Once the GI tract is removed the kidneys, adrenals and gonads will be visible.
23. Verify the sex of the turtle if possible.
24. Remove kidneys, adrenals and gonads as one unit and place in 10% NBF.
25. Photograph carcass with label clearly visible.
26. Place carcass in a labeled bag and freeze at -20°C.

Standard Operating Procedure: Packed Cell Volume
SOP#9

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining packed cell volume following collection of blood.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC.

4. Materials and Equipment

1. Blood filled microtainers or vacutainers
2. Blood sample inventory datasheet
3. Pre-labeled micro-hematocrit capillary tubes
4. Micro-hematocrit capillary tube sealant such as plasticine or critoseal
5. Micro-hematocrit centrifuge
6. Micro-hematocrit card reader

5. Procedure

1. Place a micro-hematocrit capillary tube in a blood filled microtainer, vacutainer, or microcentrifuge tube and fill the capillary tube 2/3 to 3/4 full via capillary action. Do not overfill. Use a heparin tube if the blood has not already been exposed to heparin. Alternatively, carefully place the touch of the tip of a heparinized micro-hematocrit capillary tube on a well-rounded drop of blood formed at the blood collection site after a blood sample has been collected via a syringe. A heparinized micro-hematocrit tube can be recognized by a red ring on the tube. Do not allow the capillary tube to puncture the skin. Hold the tube at a downward angle and avoid air bubbles. Fill the tube 2/3 to 3/4 full via capillary action and do not overfill. Invert the tube to ensure the blood comes into contact with the heparin.
2. Fill one end of the tube with a sealant such as plasticine or critoseal. Hold the clay perpendicular to the lab bench, holding the micro-hematocrit tube as close to the end that will be filled with clay as possible. Gently and carefully push the end of the tube into the clay 2-3 times. Excessive pressure will cause tube to break.

3. Centrifuge the sealed micro-hematocrit tubes in a hematocrit centrifuge for 2 minutes at > 12,000 g
4. Measure PCV with a card reader.

Standard Operating Procedure: Protein (BSA) assay
SOP#10

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining the protein content in cells and tissues

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC.

4. Materials and Equipment

1. Micro BCA Protein Assay Kit (#23235); working range 2-40 µg/ml
2. 96-well microplate with plastic cover
3. Molecular Devices 96-well Spectrophotometer and computer
4. Pipettes and sterile pipette tips
5. Incubator (for 37°C)
6. 15 mL and 50 mL sterile plastic tubes

5. Procedure

1. **Preparation of Diluted Albumin (BSA) standards.** Refer to the manufacturers protocol to prepare a range of 9 dilutions from 200 to 0 µg/ml BSA.
2. **Preparation of the Working Reagent (WR).** The working reagent must be made fresh each morning of the assay, refer to the manufacturers protocol to make the appropriate volume WR for each set of assays, using 25 parts of Reagent MA, 24 parts of Reagent MB and 1 part of Reagent MC.
3. **Microplate Procedure.** Refer to the manufacturers procedure. In summary pipette 150 µl of each standard or sample to a well (ideally in triplicate if not in duplicate), add 150 µl of WR. Mix the plate for 30 seconds on the spectrophotometer. Cover with sealing tape and incubate at 37°C for 2 hours.
4. Cool plate to room temperature before reading at 562 nm on the spectrophotometer. To read, open the Molecular devices imaging software and select the default protein assay, BCA method protocol, set-up plate template to determine where the standards, samples and blanks are placed.
5. Save data file and place data in Excel and subtract the average blank absorbance reading from the standard and unknown samples.

6. Prepare a standard curve by plotting the average blank corrected 562 nm reading for each BSA standard versus it's concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample. NOTE: use a best-fit polynomial equation rather than a linear equation for the standard curve.

Standard Operating Procedure: Tissue Homogenization procedure (for liver)
SOP#11

1. Scope and Applicability

The purpose of this document is to describe the procedure for preparing lysate (supernatant) from liver tissue samples.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC.

4. Materials and Equipment

1. Glass homogenization tubes with pestle
2. Pre-labeled 1.5 mL tubes
3. Phosphate buffered saline, pH 7.4
4. Ice
5. Weighing balance
6. Centrifuge

5. Procedure

1. Remove the liver samples (stored in cryovials) from the -80°C freezer and place on ice.
2. Weigh the samples and record weights
3. Depending upon specific assay (SOP 12a/13a or 14a) use the volume of buffer suggested and add liver and buffer to the homogenization tube, keep on ice.
4. Using pestle attached to a drill (on a low speed setting) homogenize sample on ice for 1-2 min until an even homogenate is formed.
5. Add the homogenate to one or two (number will depend on the assay being used) labeled eppendorf tubes and spin for 5 min at 4°C at 3,000 g.
6. Remove supernatant and place in new labeled eppendorf tubes and keep on ice or place in N2, then -80°C if not assayed immediately, the pellet can be discarded.

Standard Operating Procedure: Glutathione quantitation (tGSH and GSSG) in blood cells (SOP#12a) and liver tissue (SOP #12b)

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining total glutathione (tGSH) and oxidized glutathione (GSSG) levels in whole blood and liver samples. The assay employs a pyridine derivative (PD) as a thiol-scavenging reagent representing the most effective method compared to previously described methods using N-ethylmaleimide (NEM) or 2-vinylpyridine (VP). The PD reacts quickly with GSH but does not interfere with glutathione reductase (GR) activity. The quantitation of tGSH (i.e., GSH and GSSG) employs an enzymatic method where the reaction of GSH with Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) gives rise to a product that is quantified using spectroscopy at 412 nm. The reaction measures the reduction of GSSG to GSH and the rate of reaction is proportional to the GSH and GSSG concentration.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC and specific training on the instrumentation and procedures within.

4. Materials and Equipment

1. Molecular devices SpectraMax 96-well plate reader, computer with ImagePro
2. Microcentrifuge
3. Microcentrifuge tubes
4. Adjustable micropipettes and multichannel pipettes (10-100 µL) and tips
5. Butylated hydroxytoluene (BHT) made as 0.5 M BHT in DMSO
6. Sterile DI water
7. Assay kit GT40 from Oxford containing (keep components at 4°C unless noted otherwise):
   a. Assay buffer
   b. Standard: 10 µM GSSG standard solution (store at -20°C)
   c. Scavenger: thiol scavenger to keep GSSG in its oxidized form
   d. DTNB: lyophilized 5, 5'-dithiobis-2-nitrobenzoic acid
   e. 5% metaphosphoric acid (MPA); used to deproteinate samples
   f. Reductase: recombinant glutathione reductase (store at -20°C)
   g. NADPH: lyophilized B-nicotinamide adenine dinucleotide phosphate (store at -20°C)

5. Sample Preparation

1. The whole blood and liver samples should be removed from the -80°C just before use and placed on ice. Whole blood is used directly in this assay, the liver supernatant should be prepared as detailed in SOP # 11 with the addition of BHT during homogenization at a final concentration of 5 mM.

6. Sample Assay Procedure

1. General Information:

- Do NOT leave the reagent bottles open. Replace caps as soon as the desired volume is taken out. Use aseptic techniques when opening and dispensing reagents.

- Store the components of the kit at the temperatures specified on the labels (see above).

2. Reagent Preparation:

- **NADPH:** reconstitute contents of vial with 500 µL assay buffer. Add this to 5.5 ml assay buffer and vortex, keep on ice.
- **Reductase:** add 30 µL reductase to 6 ml assay buffer, leave on ice.
- **DTNB:** reconstitute contents of the vial with 500 µL assay buffer. Add this to 5.5 ml assay buffer and vortex, keep at room temperature.
- **Standard Curve preparation:** add the indicated volume of assay buffer and 10 µM GSSG standard stock according to the table below. Keep tubes on ice.

3. Sample Preparation for whole blood assay (SOP #12a):

- *GSSG SAMPLE:*
  - Add 30 µL scavenger to microfuge tube
  - Add 100 µL whole blood as soon as defrosted on ice to the tube and gently mix, keep at RT for 5-10 minutes.
  - Add 270 µL ice-cold 5% MPA and vortex
  - Centrifuge at 1,000 x g for 10 minutes at 4°C
  - Take 50 µL of the supernatant to 700 µL assay buffer in a new tube and mix and keep on ice until use. The pellet can be discarded.
- *GSH sample:*
  - Add 50 µL whole blood to 350 µL of ice cold 5% MPA and vortex mix
  - Centrifuge at 1,000 x g for 10 minutes at 4°C
  - Take 25 µL of the supernatant to 1500 µL assay buffer in a new tube and mix and keep on ice until use. The pellet can be discarded.

Standard	GSH conc. (µM)	GSH conc. (µM)	Vol. of Assay Buffer (µL)	Vol. of 10 µM GSSG stock (µL)	Final vol. (µL)
S7	3.0	1.5	850	150	1000
S6	2.0	1.0	900	100	1000
S5	1.5	0.75	925	75	1000
S4	1.0	0.50	950	50	1000
S3	0.5	0.25	975	25	1000
S2	0.25	0.125	987.5	12.5	1000
S1	0.10	0.05	995	5	1000
B0	0	0	1000	0	1000

4. **Sample Preparation for liver tissue assay (SOP #12b):**
   - *GSSG SAMPLE:*
     - Add 30 µL scavenger to microfuge tube
     - Add 100 µL liver preparation supernatant as soon as defrosted on ice to the tube and gently mix, keep at RT for 5-10 minutes.
     - Add 270 µL ice-cold 5% MPA and vortex
     - Centrifuge at 1,000 x g for 10 minutes at 4°C
     - Take 50 µL of the supernatant to 700 µL assay buffer in a new tube and mix and keep on ice until use. The pellet can be discarded.
   - *GSH sample:*
     - Add 50 µL of the liver preparation supernatant to 350 µL of ice cold 5% MPA and vortex mix
     - Centrifuge at 1,000 x g for 10 minutes at 4°C
     - Take 25 µL of the supernatant to 1500 µL assay buffer in a new tube and mix and keep on ice until use. The pellet can be discarded

5. **Performing the Assay (for both whole blood and liver supernatants):**
   - Add 50 µL of standards, samples, or blank to the corresponding wells on the microplate.
   - Add 50 µL DTNB solution to each well
   - Add 50 µL reductase solution to each well
   - Mix the wells by taping the plate and incubate at room temperature for 5 minutes.
   - Ad 50 µL NADPH solution to each well
   - Place the plate in the spectramax and using the kinetic function record the change of absorbance at 412 nm by taking readings every minute for 10 minutes

6. **Calculations:**
   - Rate determination (mean V): The change in absorbance (or optical density (OD) is a linear function of the GSH concentration in the reaction mixture;

Where: OD = slope x minutes + intercept

- **Calibration curves**: an 8 point calibration curve for both GSH and GSSG should be prepared (see Table above). For each concentration of GSH / GSSG calculate the net rate i.e., the net rate is the difference between sample or standard and the blank rate (B0). For GSH use the GSH standards 0, 1.0, 2.0 and 3 µM. For GSSG use the 0, 0.05, 0.125 and 0.25 µM GSSG data points.
- **GSH and GSSG concentration in samples**:
  The general form of the regression equation describing the calibration curve is:

  Net rate = slope X GSH + Intercept

  Therefore, to calculate the tGSH or GSSG concentration from the GSHt or GSSG calibration curves, using:

$$\text{GSHt (or GSSG)} = \frac{\text{net rate change intercept}}{\text{slope}} \times \text{dilution factor}$$

- **GSH/GSSG ratio:** the GSH/GSSG ratio is calculated by dividing the difference between the GSHt and the GSSG concentration by the concentration of GSG using:
-

$$\text{Ratio} = \frac{\text{GSHt} \times 2(\text{GSSG})}{\text{GSSG}}$$

7. Data Management and Records Management:

- Print outs of raw data will be maintained in a file named "Total antioxidant power assay" in the locked designated room. Raw data electronic files will be maintained on the SpectraMax computer and also copied to all hard and thumb drives for the test 350 and 351 work.
- All electronic worksheets (.xls/.doc) generated to convert the raw data into the final format will also be copied onto all multiple storage devices for the 350 and 351 work.
- All final quantitative results will be entered onto the sample inventory/data summary sheets to check for QAQC endpoints (e.g., acceptable CV for replicates etc.).

8. Quality control and Quality Assurance:

- All standards and samples will be run in duplicate or triplicate (IDEAL).
- Check that the standard curves generated fall within the normal ranges and that r2 values are >95%.
- Check that the samples fall within the standard curve (i.e., the dynamic range of the assay i.e., 1.0 – 3.0 µM GSHt or 0.05 – 0.25 µM GSSG); re-

run any samples that do not fall into this range by diluting in 1x phosphate buffered saline (PBS).

- Check that the % coefficient of variation (%CV) of replicate values (can only really do this if triplicates are used) is no greater than 10%. Re-run any samples with a CV of >10%.

Standard Operating Procedure: Lipid peroxidation assay (TBARS) for plasma (SOP #13)

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining the extent of lipid peroxidation in liver tissue samples (in terms of total MDA equivalents). The assays utilize the Lipid Peroxidation TBARS Colorimetric Assay Kit (FR40) from Oxford Biomedical Research, Oxford, MI modified for the FR45 fluorescent plate method. TBARS are present in biological specimens and include lipid hydroperoxides and aldehydes which increase in concentration in response to oxidative stress. TBARS assay values are reported in MDA equivalents. The TBARS assay utilizes the reaction of the chromogenic reagent,2-thiobarbituric with malondialdehyde (MDA). One molecule of MDA reacts with two molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with a maximal absorbance at 532 nm.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC and specific training on the instrumentation and procedures within.

4. Materials and Equipment

1. Molecular devices SpectraMax 96-well plate reader, computer with ImagePro
2. Microcentrifuge
3. Microcentrifuge tubes
4. Water bath set to 65°C
5. Adjustable micropipettes and multichannel pipettes (10-100 µL) and tips
6. 96-well BLACK microplate (in the FR45 kit)
7. Butylated hydroxytoluene (BHT) made as 0.5 M BHT in DMSO
8. Sterile DI water
9. Saturated ammonium sulfate solution in DI
10. Saturated trichloroacetic acid (TCA) solution (in DI)
11. Assay kit FR40 from Oxford containing:
    a. Indicator: 2-Thiobarbituric Acid
    b. Acid Reagent: 10% acid solution in DMSO
    c. Malondialdehyde (MDA) Standard: 10 mM Malonaldehyde Tetrabutylammonium Salt

5. Sample Preparation

1. The plasma samples should be removed from the -80°C just before use and placed on ice.

6. Sample Assay Procedure

1. General Information:

- Do NOT leave the reagent bottles open. Replace caps as soon as the desired volume is taken out.

- Sample blanks for each sample are recommended for this assay, this should contain the sample plus the acid reagent alone (no TBA indicator).

- The acid reagent freezes at temperatures 15°C or colder, before use remove it from the kit at 4°C for an hour to reach RT and thaw.

2. Sample Preparation:

- *ACID REAGENT:* Remove from 4°C and allow to sit at room temperature for an hour to thaw before use.
- *INDICATOR SOLUTION:* Add 10 ml of the Acid reagent to the powdered contents of one vial of indicator and shake and mix well until completely dissolved.
- *20 µM MDA standard stock:* Dilute the 10 mM MDA standard 1:500 in DI water (i.e., 20 µL of the 10 mM MDA and 9.98 mL of DI) to obtain a 20 µM MDA standard stock solution. Prepare the set of seven standards as described in the following table (see page 40). NOTE: MDA is provided in the MDA-TBA salt in a slightly basic buffer as MDA itself is not stable. When mixed with acid Indicator solution, the MDA-TBA molecule is acidified and generates MDA quantitatively.
- *PLASMA preparation:* The plasma should be deproteinated before use. To 100 µL plasma add 2.5 µL BHT stock solution and 157.5 µL 1 x PBS (pH 7.4), then add 20 µL saturated TCA solution (in DI) and 20 µL of saturated ammonium sulfate solution (in DI). Vortex mix and centrifuge samples at 15,000 x g for 10 min. Remove the supernatant to a new tube and use for the assay. The pellet can be discarded.

3. Performing the Assay – for Total MDA:

1. ***Preparation of Standards and Samples:***
2. Add 200 µL of each of the standards or samples to a microcentrifuge tube.
3. Add 200 µL of Indicator solution to each tube and vortex mix.
4. ***Preparation of Sample Blanks:***
5. Add 100 µL of each of the samples to a microcentrifuge tube.
6. Add 100 µL of Acid reagent to each tube and vortex mix.

7. Incubate all standard, sample and blank tubes in the water bath at 65°C for 45 minutes
8. Transfer two 150 µl aliquots of each standard/sample (in duplicate) or blanks (singly) to the designated wells on the microplate.

- Read the plate at 532 nm Ex and Em

Standard	MDA conc. (µM)	Vol. of DI (µL)	Vol. of 20 µM stock (µL)
S0	0	400	0
S1	0.5	390	10
S2	1.0	380	20
S3	2.5	350	50
S4	5.0	300	100
S5	10	200	200
S6	15	100	300
S7	20	-	400

4. Calculations:

9. Plot the absorbance at 586 nm (A_{586}) of each standard versus it's MDA concentration and perform a linear regression analysis:

Where: $A_{586} = m[MDE + HAE] + b$

A_{586} = absorbance at 586 nm
m = slope of the standard curve
[MDE + HAE] = µM concentration of (MDE + HAE) in the sample
b = y-intercept

- Calculate the concentration of analyte in each unknown using:

[MDE + HAE] = {(A456-b) / m} x dilution factor

5. Data Management and Records Management:

- Print outs of raw data will be maintained in a file named "Total antioxidant power assay" in the locked designated room. Raw data electronic files will be maintained on the SpectraMax computer and also copied to all hard and thumb drives for the test 350 and 351 work.
- All electronic worksheets (.xls/.doc) generated to convert the raw data into the final format will also be copied onto all multiple storage devices for the 350 and 351 work.

- All final quantitative results will be entered onto the sample inventory/data summary sheets to check for QAQC endpoints (e.g., acceptable CV for replicates etc.).

6. Quality control and Quality Assurance:

- All standards and samples will be run in duplicate or triplicate (IDEAL).
- Check that the standard curves generated fall within the normal ranges and that r2 values are >95%.
- Check that the samples fall within the standard curve (i.e., the dynamic range of the assay i.e., 1.0 – 20 μM); re-run any samples that do not fall into this range by diluting in 1x phosphate buffered saline (PBS).
- Check that the % coefficient of variation (%CV) of replicate values (can only really do this if triplicates are used) is no greater than 10%. Re-run any samples with a CV of >10%.

Standard Operating Procedure: Lipid peroxidation assay for liver (SOP #14)

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining the extent of lipid peroxidation in liver tissue samples. The assays utilize the Lipid Peroxidation Microplate Assay Kit (FR22) from Oxford Biomedical Research, Oxford, MI. The assay utilizes the reaction of the chromogenic reagent, N-methyl-2-phenylindole (R1), with malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) at 45°C. one molecule of either MDA or HAE reacts with two molecules of Reagent R1 to yield a stable chromophore with a maximal absorbance at 586 nm.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC and specific training on the instrumentation and procedures within.

4. Materials and Equipment

1. Molecular devices SpectraMax 96-well plate reader, computer with ImagePro
2. Microcentrifuge
3. Water bath set to 45°C
4. Adjustable micropipettes and multichannel pipettes (10-100 µL)
5. 96-well microplate
6. Butylated hydroxytoluene (BHT) made as 0.5 M BHT in acetonitrile
7. Acetonitrile
8. Sterile DI water
9. Tissue homogenization buffer (1 x PBS; pH 7.4)
10. Assay kit FR22 from Oxford containing:
    a. Reagent R1: N-methyl-2-phenylindole in acetonitrile
    b. Reagent R2: methanesulfonic Acid (MSA)
    c. Malondialdehyde (MDA) Standard (NOTE: the MDA is provided as an acetal i.e., 1,1,3,3-tetramethoxypropane (TMOP) in Tris-HCl as the aldehyde is not stable. The TMOP is hydrolyzed during the acid incubation step at 45°C thereby generating MDA.
    d. Reagent dilutant: Ferric iron in methanol

5. Sample Preparation

1. The liver tissue is homogenized in 5-10 ml of ice cold PBS buffer (pH 7.4) containing 5 mM BHT (which prevents sample oxidation) per gram of liver tissue. The homogenate is centrifuged at 3,000 x g for ten minutes at 4°C. The supernatant is aliquoted into multiple eppendorf tubes, one of which is used for this assay (and left over frozen for the protein assay), any remaining should be frozen in N2 and stored at -80°C immediately. Dilute the homogenate if needed with the homogenization buffer. NOTE: the pellet from the centrifugation step is discarded.

6. Sample Assay Procedure

1. General Information:

- Do NOT leave the reagent bottles open. Replace caps as soon as the desired volume is taken out.
- Do NOT allow the capped reagent bottles to sit at room temperatures for any extended periods of time.
- Reagent R2, methanesulfonic acid (MSA) will freeze at temperatures 19°C or colder, before use remove it from the kit at 4°C for a couple hours to reach RT and thaw.

2. Sample Preparation:

- Dilute the 10 mM MDA standard 1:500 in DI water (i.e., 20 µL of the 10 mM MDA and 9.98 mL of DI) to obtain a 20 µM MDA standard stock solution. Prepare the set of seven standards as described in the following table (see page 41):

3. Performing the Assay:

1. Dilute Reagent R1 3:1 with the dilutant provided (i.e., 12 mL of R1 and 4 mL of diluent).
2. Add 100 µL of each of the standards or samples to a microcentrifuge tube.
3. Add 325 µL of diluted Reagent R1 to each tube and vortex mix.
4. Add 75 µL of Reagent R2 (MSA) to each tube and vortex mix well.
5. Incubate all tubes in the water bath at 45°C for 60 minutes
6. Centrifuge the samples at 15,000 x g for 10 minutes to obtain a clear supernatant.
7. Transfer three (two minimum) 150 µl aliquots of each standard/sample to the designated wells on the microplate.
8. Centrifuge briefly up to 2,000 rpm for a few seconds to remove bubbles.
9. Read the plate at 586 nm

Standard	Vol. of DI water (µL)	Vol. of 20 µM MDA stock (µL)	MDA concentration (µM)
BLK	200	0	0
S1	190	10	1.0
S2	175	25	2.5
S3	150	50	5.0
S4	100	100	10.0
S5	50	150	15.0
S6	0	200	20.0

4. Calculations:

10. Plot the absorbance at 586 nm (A_{586}) of each standard versus it's MDA concentration and perform a linear regression analysis:

Where: $A_{586} = m[MDE + HAE] + b$

A_{586} = absorbance at 586 nm
m = slope of the standard curve
[MDE + HAE] = µM concentration of (MDE + HAE) in the sample
b = y-intercept

- Calculate the concentration of analyte in each unknown using:

  [MDE + HAE] = {(A456-b) / m} x dilution factor

5. Data Management and Records Management:

- Print outs of raw data will be maintained in a file named "Total antioxidant power assay" in the locked designated room. Raw data electronic files will be maintained on the SpectraMax computer and also copied to all hard and thumb drives for the test 350 and 351 work.
- All electronic worksheets (.xls/.doc) generated to convert the raw data into the final format will also be copied onto all multiple storage devices for the 350 and 351 work.
- All final quantitative results will be entered onto the sample inventory/data summary sheets to check for QAQC endpoints (e.g., acceptable CV for replicates etc.).

6. Quality control and Quality Assurance:

- All standards and samples will be run in duplicate or triplicate (IDEAL).
- Check that the standard curves generated fall within the normal ranges and that r2 values are >95%.

- Check that the samples fall within the standard curve (i.e., the dynamic range of the assay i.e., 1.0 – 20 μM); re-run any samples that do not fall into this range by diluting in 1x phosphate buffered saline (PBS).
- Check that the % coefficient of variation (%CV) of replicate values (can only really do this if triplicates are used) is no greater than 10%. Re-run any samples with a CV of >10%.

Standard Operating Procedure: Total antioxidant assay (TA) for liver (SOP#15a) and plasma (SOP#15b)

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining the total antioxidant power in liver cells or plasma.

Summary of method: The assays are run using the Oxford Biomedical Research (Oxford, MI) Total Antioxidant Power Kit (Item Number: TA02). The assay utilizes the reduction potential of the sample (or standard) to reduce copper i.e., Cu 2 + to Cu 1 +. This reduced form of copper will selectively for a stable 2:1 complex with the chromogenic reagent which has an absorption maximum at 450 nm. A calibration curve is generated using known concentrations of Trolox, therefore, the data is expressed in terms of mM Trolox equivalents.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC. All personnel will be trained on the specific procedures and instrumentation by the project manager.

4. Materials and Equipment

1. Molecular Devices spectramax with softmax pro software
2. Microcentrifuge
3. Adjustable single and multi-channel pipettes (10-1000 µL)
4. Plasma sample inventory datasheet and TA sample inventory datasheet and plate set-up sheet
5. Pre-labeled eppendorf tubes
6. Absolute ethanol (200 proof)
7. Tissue homogenization buffer for liver tissues (Phosphate Buffered Saline, pH 7.4; see SOP 11a).
8. Liver tissue or plasma samples stored at -80°C until assay
9. Ice bucket and Ice
10. Homogenization tube and pestle and drill
11. Oxford Biomedical kit TA02 containing:
    - 8a. Dilution buffer
    - 8b. Copper solution
    - 8c. Stop solution

8d. Trolox standard
8e. 96-well microplate

12. Deionized water
13. Latex or nitrile gloves

5. Sample Preparation Procedure (SOP 15a: Liver tissue)

1. Remove samples from -80°C and record weight of tissue in TA sample inventory and calculate the 7x volume of 1x PBS buffer to add, record the volume added in (2).
2. Add tissue to homogenization tube (keep on ice), containing x7 volume of ice-cold PBS (pH 7.4) per gram of tissue. Homogenize the tissue on ice.
3. Pour homogenate into one or more pre-labeled 1.5 or 2 ml eppendorf tubes and centrifuge at 3,000 g for 10 minutes at 4°C.
4. Remove the supernatant for the assay and store samples on ice. A minimum of 700 µL is required for the TA02 assay (with left over at end stored at -80°C for the protein assay) and additional should be placed in N2 and stored at -80°C.
5. Dilute samples as needed with the 1x PBS solution (homogenization solution used in (1).
6. NOTE: the pellet from the centrifugation step should be discarded.

6. Sample Preparation Procedure (SOP 15b: Plasma samples)

1. Remove samples from -80°C and place on ice, once thawed samples should be vortexed and are now ready for the sample assay procedure.

7. Sample Assay Procedure (for both SOP 15a: Liver tissue and SOP 15b: Plasma samples)

1. NOTE: before starting the assay equilibrate the dilution buffer, copper solution and stop solution to RT 30 minutes prior to assaying.
2. **Preparation of standards:** The provided Trolox solution is under vacuum and so using a sterile syringe and needle add 2.0 mL of absolute (200 proof) ethanol directly to the Trolox Standard vial through the unopened rubber stopper. NOTE: I would first add 1 mL EtOH and then remove 1 mL of air from the tube. Then add the second 1 mL. Vortex the vial for 30-60 seconds, or until the standard is completely dissolved, the standard stock is now 2.0 mM. Prepare a set of standards using the following table using 2 ml tubes (NOTE: for triplicate standards a volume of >600 µL is needed):

Standard	Trolox Final Conc. (mM)	DI water (µL)	Transfer volume (µL)	Transfer source	Final volume (µL)
S5	2.0	NONE	2000	2 mM STOCK	1200
S4	1.0	800	800	S5	800
S3	0.5	800	800	S4	800
S2	0.25	800	800	S3	
S1	0.125	800	800	S2	
Blk	0	800	NONE		800

3. **Performing the assay:** dilute both the samples (plasma or liver supernatants) and the standards 1:40 in the provided dilution buffer (e.g., to pre-labeled tubes add 780 µL of dilution buffer and place 20 µL plasma/liver homogenate or standard into the tubes), vortex mix.
4. Place 200 µL of the diluted standards or samples in triplicate into each well in the 96-well plate. Refer to the plate assay template sheet.
5. Place the 96-well plate in the centrifuge and BRIEFLY spin to remove bubbles (i.e., turn on and after a few seconds reaching approx. 1800 rpm turn the centrifuge off).
6. Read the plate at 450 nm for a reference plate measurement, save the file with the file name 'pre-read'.
7. Add 50 µL of the copper solution to each well and incubate for 3 minutes at RT.
8. Add 50 µL stop solution to each well and re-spin the plate briefly as outlined in (12) to remove bubbles.
9. Re-read the plate at 450 nm and save the file with the file name 'post-read'.
10. Save files on the Spectramax computer, and to all other storage devices.
11. **Calculations:** Calculate the net absorbance by subtracting the absorbance readings from the first plate read (pre-read) from those of the second plate read (post-read).
12. Plot the net absorbance of the standards versus their Trolox concentration and perform a linear regression analysis:

Where: $A_{450} = m[\text{trolox}] + b$

A_{450} = absorbance at 450 nm
m = slope of the standard curve
[trolox] = mM concentration of Trolox equivalents
b = y-intercept

13. Calculate the concentration of Trolox equivalents in each sample using:

    [Trolox] = {(A450-b) / m} x dilution factor

14. Values can also be expressed as µM copper reducing equivalents (CRE) by multiplying the mM equivalents of Trolox by 2189.

15. Data Management and Records Management:

16. Print outs of raw data will be maintained in a file named "Total antioxidant power assay" in the locked designated room. Raw data electronic files will be maintained on the SpectraMax computer and also copied to all hard and thumb drives for the test 350 and 351 work.
17. All electronic worksheets (.xls/.doc) generated to convert the raw data into the final format will also be copied onto all multiple storage devices for the 350 and 351 work.
18. All final quantitative results will be entered onto the sample inventory/data summary sheets to check for QAQC endpoints (e.g., acceptable CV for replicates etc.).

19. Quality control and Quality Assurance:

20. All standards and samples will be run in duplicate or triplicate (IDEAL).
21. Check that the standard curves generated fall within the normal ranges and that r2 values are >95%.
22. Check that the samples fall within the standard curve (i.e., the dynamic range of the assay i.e., 0.125 – 2.0 mM); re-run any samples that do not fall into this range by diluting in 1x phosphate buffer.
23. Check that the % coefficient of variation (%CV) of replicate values (can only really do this if triplicates are used) is no greater than 10%. Re-run any samples with a CV of >10%.

Standard Operating Procedure: COMET assay for blood cells (SOP#16a) and liver tissue (SOP#16b)

1. Scope and Applicability

The purpose of this document is to describe the procedure for determining the extent of DNA single strand breaks (DNA damage) using the single cell gel electrophoresis (or COMET) assay.

2. Health and Safety

Workers should be familiar with procedures for biohazardous waste disposal and cleanup.

3. Personnel Qualifications

All persons involved in laboratory work will have completed all necessary training including Institutional IACUC and training on the specific protocol and instrumentation of this assay.

4. Materials and Equipment

1. Adjustable single-channel pipettes (10-1000 µL) and sterile tips
2. Blood and liver tissue sample inventory datasheet and COMET sample inventory datasheet
3. Pre-labeled eppendorf tubes
4. Pre-labeled glass slides
5. Aluminum trays on ice
6. Ice buckets and ice
7. Pre-labeled and etched glass slides coated in dried 1% normal melting point agarose (NMA)
8. Labeled slide storage box in ziplock bags containing desiccant
9. Ice-cold MeOH
10. Tissue homogenization buffer for liver tissues and diluting buffer for whole blood (i.e., HBSS, pH 7.4)
11. Petrifilm
12. Water bath at 36°C
13. 0.75% Low melting point agarose (LMPA) in tubes in water bath at 36°C
14. Dark room with yellow lighting
15. Freshly made 1 x Ice-cold lysing solution in slide storage box
16. Freshly made 1 x Electrophoresis buffer
17. Ice-cold neutralization buffer
18. Disposable 70 µM nylon sieves
19. Sterile Teflon coated single edged razor blades
20. Sterile forceps
21. Large electrophoresis chamber and power supply

22. Glass slide staining boxes
23. DMSO
24. Triton X 100
25. Ethidium bromide stock solution
26. EDTA (disodium salt)
27. N-Laurylsarcosine
28. Hanks Buffered Salt Solution
29. NaCl
30. NaOH
31. 0.2 µM sterile filters
32. Low and normal melting point agarose
33. Tris base (Sigma T-8524)
34. No. 1 microscope slides (22 x 50 mm)
35. Ethidium bromide solution (20 µg/ml filtered via 0.2 µM filter); stored in dark at 4°C.
36. Epiflourescent microscope with black and white digital camera
37. KOMET 5.0 image analysis system

5. Preparation of samples: whole blood cells (SOP # 16a)

5 µL of whole blood is placed in a microcentrifuge tube containing HBSS + HEPES (pH 7.6), mix and immediately use 10 µL of this solution in the COMET procedure below.

6. Preparation of samples: liver cells (SOP # 16a)

Place a small amount of freshly collected liver (about 2 mm-3 mm in radius) on a glass microscope slide on ice containing two to three droplets (around 100 µL-150 µL) of HBSS and HEPES solution and mince into small pieces, pipette the solution through a 70 µM filter into a eppendorf tube and repeat. Centrifuge the solution at 1,500 x g and use the bottom pellet containing part for the assay below.

7. Preparation of stock solutions and glass slides and fresh working solutions made on day of assay

STOCK Lysing Solution (10 mM Tris, 200 mM NaCl, 100 mM Na_2EDTA, N-Laurylsarcosine)

Add 37.2 g EDTA, 146.1 g NaCl and 1.2 g Tris base to 600 mL DI water and add ~3–4 g NaOH to get these chemicals into solution. When in solution pH to 10 using NaOH pellets (about 8 g total). Add 10 g N-Laurylsarcosine and q.s. to 890 mL with DI water, filter sterilize using 0.2 µM filter and keep stock at RT.

STOCK 200 mM disodium EDTA

Add 37.22 g EDTA to 500 mL DI water, store at RT.

STOCK 10 M NaOH

Caution make in fume hood, use goggles, lab coat, gloves, as this is a highly exothermic reaction. Add 40 g NaOH to 100 mL DI water, store at RT.

Electrophoresis Buffer (2 L, pH>12; store at 4°C)

Add 40 mL NaOH stock and 10 mL EDTA stock, q.s. 2 L with DI water and store at 4°C until use.

Lysing Solution (110 ml; Store at 4°C; i.e., stock lysing solution with 10% DMSO and 1% Triton)

Add 11 mL DMSO and 1.1 mL Triton x-100 and q.s. 110 mL with stock lysing solution, mix well, add to slide staining tray and store in fridge.

Neutralization Buffer (store at 4°C)

Add 48.5 g Tris base to 800 mL DI water, adjust pH to 7.5 with HCl then q.s. to 1000 mL with DI water, store in the fridge.

HBSS + Hepes (pH 7.6)

Add 0.6507 g Hepes in 250 mL HBSS, aerate and pH to 7.6; filter sterilize and store at room temp.

Ethidium Bromide (store in the dark at 4°C; *use nitrile gloves when handling!!!!*)

Need to do a 1:5000 dilution of stock EtBr i.e., make a 1:100 dilution: 100µL EtBr + 9.9 mL DI water, then make a 1:5000 dilution: 1 mL of 1:100 diluent + 49 mL DI water. NOTE: If drying down slides, bring EtBr up in Hepes + HBSS.

1% NMPA

1 g NMPA in 100 mL PBS, boil to melt and use immediately to coat glass slides.

0.75% LMPA

0.075 g LMPA→ 10 mL HBSS + Hepes buffer, boil to melt and store at 37°C before use.

Coating of glass microscope slides

Use unfrosted, uncleaned microscope slides, using a diamond pen etch numbers in the upper right hand of the slide and then etch "**X's**" across lower 3/4 of slide surface. Dip slides in one at a time about 3/4 of total slide length in the hot 1% NMA. Wipe the back of the slides and place on aluminum foil at 37°C in a drying oven. Once dry slides can be stored at RT in a ziplock bag containing a slide tray and desiccant.

8. Assay procedure: for both blood and liver cells:

- Carry out the assay in the dark under minimal light (including sample preparation above) and cover all slides at all times once prepared with foil.
- Remove coated slides from drying oven and place on a paper towel ready for use.
- Make single cell suspension from liver and blood as detailed above.
- Pipette 10μL of cell suspension (mix up and down before one that's used etc.) onto Parafilm and quickly add to mix with 100μL of 37°C LMPA from water bath
- -Pipette 100μL of mixture onto slide (remember quickly down the length of the left slide and place coverslip over (drop from left side down to spread in even thin layer etc.).
- -Allow to solidify on ice /metal block in dark for 5-10 minutes (NOTE: all times in protocol log sheet).
- Once the agarose has solidified, carefully remove coverslip (remember feel if there's slight resistance first so you know it's solidified etc.).
- Place slide in the freshly made lysing solution (slide box at 4°C); remember you can put 20 of them back to back, be very careful to remember which side has the agarose and which doesn't etc.), place for 1 hour (or more) at 4°C in the dark.
- Allow cells to lyse for at least 1 hour but not more than 24 hours (there will be some precipitate in the bottom of the lysing chamber – this is normal) – NOTE all times in the sample log sheet etc.
- After cells have been in lysing solution, remove carefully and rinse with DI water (use squeezy bottle gently rinse front and back of slides). Place on tissues / rack until all done etc.
- While cells are lysing remove the freshly made electrophoresis buffer from the fridge and put into the electrophoresis chamber, connect power supply and set a constant voltage to 25V, adjust the electrophoresis buffer height to achieve ~ 300 milliamps (NOTE: adding buffer increases amps, removing buffer reduces amps. Turn off to place slides in chamber.
- Once cells have finished the lysing and rinsing steps above, quickly place slides in the electrophoresis chamber (slide in at an angle and it's best to get them all in as soon together as possible (16 max per run), once all slides are in the chamber, start timing (NOTE: the exact positions can be moved during this time so they are in straight lines etc. Keep slides in electrophoresis solution for 10 minutes.
- After 10 minutes turn on the power supply (NOTE: buffer volume can be quickly adjusted if needed to maintain the 300 mA start), electrophorese the slides for 10 minutes, after which disconnect power supply.

- Take out the slides, let most of the excess buffer drip off and then rinse the slides in neutralization buffer i.e., let sit for 10 minutes in solution (in a glass slide tray, use solution once for 20 slides etc.). Drain the buffer from slides and place slides on paper towels agarose side up.

- Place slides in ice cold ethanol or methanol for 5 minutes (place in a slide staining tray at 4°C is the easiest method).

- Remove the slides and lay out on aluminum foil to dry in the dark at RT for 30 min – 1 hour.

- When dry, store in a microscope slide box with desiccant in the dark at RT.

- To rehydrate to analyze the slides: Pipette 50µL of Ethidium Bromide diluted with Hepes-buffered HBSS and place a coverslip on slide. Using the epifluorescent microscope under green light, using 20x objective (total x400 magnification), use Komet Software by Kintetic Imaging, UK to score 50 cells per slide in a random order, measure DNA tail %, tail length, and tail moment.

Attachment 1:

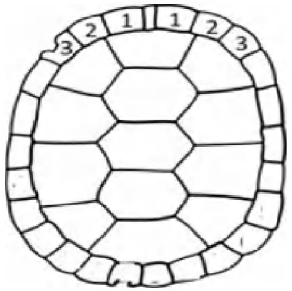
Additional Datasheets

E-tube Insertion Surgery form
Daily health monitoring
Environmental monitoring
Dosing datasheet
Blood sample inventory
Necropsy
Photolog
PCV

Etube Insertion Surgery Form

Turtle ID		Date			
Species	*T. scripta*	Room Temp (F)		Table Temp	
Scute Code		Tank Temp (F)		Recovery T	
Original Tank #		Location	Cronin 2208		
Weight (g)		Time Last Feed			
SCL (mm)		DVM			
Physical Status		Recorded by			
Sex (M/F)		Photos taken			

Scute Code:



Medications:					Material:			Major Time Points:	
Drug	Time (h)	Amt (mg/kg)	Amt (ml)	Route	E tube size				Time
Propofol					Suture size-type			Initial signs:	
Meloxicam								Induction:	
Lidocaine								E tube start:	
								E tube end:	
PWD (fluids)						Time	HR	Cardio site start:	
								Cardio site end:	
Telazol								End surgery:	
								To recovery:	
								Alert:	
								Etube trial:	
								Return to tank:	

Notes:

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NOAA DWH TURTLE TOXICITY TESTING: DAILY HEALTH MONITORING DATASHEET FOR PHASE FOUR

Test ID: T04 Turtle ID #: _____ Start Date (yyyy/mm/dd): _____ Start Time: _____Handler: _____

Date (mm/dd/yr)	Time (h)	Feeding (Y/N)	Feces (Y/N)	Skin color/ health	Eye/Nose Health	Behavior normal	g-tube site observation	Water temp (F)	Oil sheen in water?	NOTES	Recorded by

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NOAA DWH TURTLE TOXICITY TESTING: DAILY ENVIRONMENTAL MONITORING DATASHEET FOR PHASE FOUR

Test ID: T04 Turtle ID #: _____ Start Date (yyyy/mm/dd): _____ Start Time: _____ Handler: _____

Date (mm/dd/yr)	Time (h)	Air Temp (F)	Water Temp (F)	Lighting on schedule (Y/N)	Feed time (h)	Feed Type (W/P)	Cleaning/ water change time (h)	NOTES	Recorded by

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NOAA DWH TURTLE TOXICITY TESTING: DOSING DATASHEET FOR PHASE FOUR

Test ID#: T04 Turtle ID #: _____ Start Date (mm/dd/yyyy): _____ Dosing group (mg/kg): _____

Dose Administrator: _____ Handler: _____ Recorder: _____ Rep. #:_____

Date (mm/dd/yr)	Time turtle taken out of tank (24 h)	Carapace length (mm)	Weight (g)	Total dose Required (mg)	Weight of in syringe to start (mg)	Weight of in syringe at end (mg)	Actual dose received (mg)	Time of dose (24 h)	Amount of feed slurry (ml)	Amount of saline received (ml)	Time placed back into tank (24 h)	Notes

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NOAA DWH TOXICITY TESTING: BLOOD SAMPLE INVENTORY DATASHEET PHASE FOUR

Test ID:_____ Date (yyyy/mm/dd): _____ Blood Collection Time: _____

Turtle ID:_____ Collection site: _____ Total blood (µL): _____ Total plasma (µL):_____

Handler: _____ Recorder: _____ Observers: _____

Blood fraction	Volume (µL)	Prep time:	Sample code	Sample label or number	Notes*
Whole Blood			PCV-Miami		
			HEM-Miami		
			WBS		
			WBS		
			WBS-NNB		
			TCT		
			GSH		
			WBS		
			WBS		
			WBS		
			DNA		
			DNA		
Plasma			BCP		
			GLDH		
			CORT		
			ARV		

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Blood fraction	Volume (µL)	Prep time:	Sample code	Sample label or number	Notes*

**Please note any indication of abnormal clotting, hemolysis or ligemia in the notes section of the datasheet*

Sample Description	Sample code
Whole Blood Smear	WBS
Whole Blood Smear stained with NMB	WBS-NMB
Microhematocrit capillary tube (PCV)	PCV-CBL
100 µL whole blood Miami (PCV)	PCV-Miami
100 µL whole blood Miami (hemoglobin)	HEM-Miami
>100 µL RBC pellet (after plasma) for GSH at CBL	GSH
20 µL whole blood prepped for DNA damage assay	DNA
Transcript (400 µL whole blood)	TCT

Sample Description	Sample code
320 µL plasma for Miami (blood chemistry panel)	BCP
100 µL plasma for Miami (GLDH)	GLDH
150 µL plasma for Cornell	CORT
Archive plasma	ARV

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NOAA DWH TOXICITY TESTING: NECROPSY DATASHEET PHASE FOUR

Test ID: _____ Date (yyyy/mm/dd): _____ Time: _____

Turtle ID: _____ Body Weight (g): _____ Body Length (mm): _____ Sex:_____

Dissector: _____ Recorder: _____ Observers: _____

Organ/tissue	Weight (g)	Gross Assessment	Sample label or number	Analysis code	Preparation (fixed or frozen)	Notes
bile (uL)						
lungs						
heart						
liver						
whole GI tract w/ spleen						
thyroid						
adrenals						
kidneys						
gonads						
muscle tissue						One hind leg

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Organ/tissue	Weight (g)	Gross Assessment	Sample label or number	Analysis code	Preparation (fixed or frozen)	Notes
carcass						

Gross assessment key; N=normal, no abnormalities noted; Ab=abnormal (specify and record digital images) Lightly shaded area on page 2 of datasheet can be used for additional samples/observations during necropsy.

Analysis	Analysis code
PAH metabolites	MET
Total glutathione (GSH + GSSG)	GSH
Lipid peroxidation	LPO
CYP enzymes	CYP

Analysis	Analysis code
DNA damage	DNA
Histology	HIS
Archive	ARV

Analysis	Analysis code

PAGE:_____ of _____.

NOAA DWH TURTLE TOXICITY TESTING: PHOTOGRAPH LOG DATASHEET FOR PHASE FOUR

Test ID: _____ Start Time: _____ Camera ID:_____ Memory card ID: _____

Photographer/affiliation: _____ Data recorder: _____

Photo file name	Turtle ID #	Date (yyyy/mm/dd)	Time (24-hr format)	Location	Subject description

PAGE:_____ of_____.

NOAA DWH TURTLE TOXICITY TESTING: PVC DATASHEET FOR PHASE FOUR

Test ID#: _____ Date (yyyy/mm/dd): _____ Analyzer: _____

Turtle ID #	Parameter	Parameter Units	Time of analysis (24hr)	Result Replicate 1	Result Replicate 2	Result Replicate 3	Average Result	Notes